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# BIOCHIMICA *et* BIOPHYSICA ACTA

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# **BIOCHIMICA ET BIOPHYSICA ACTA**

**Vol. 3 (1949)**



## THE SPECIFICITY OF THE HUMAN ERYTHROCYTE CHOLINESTERASE

by

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Earlier investigations on cholinesterase specificity were confined to horse serum<sup>16, 17</sup> and human serum<sup>19</sup>. The enzymes from these sources were shown to be non-specific in that they were capable of hydrolysing methyl butyrate and tributyrin, as well as acetyl choline.

Working with human blood, ALLES AND HAWES<sup>4</sup> demonstrated that the substrate specificity pattern of the erythrocyte cholinesterase against certain acetyl esters of choline derivatives differed from that shown by the serum cholinesterase and suggested from this and other evidence that the two enzymes are not identical. RICHTER AND CROFT<sup>14</sup>, in an investigation of blood cholinesterases from different species, confirmed the earlier suggestions regarding the non-specific nature of the human serum enzyme. The erythrocyte cholinesterase, on the other hand, was found to be inactive towards tributyrin and methyl butyrate, and these authors concluded that it was specific for acetyl choline. Evidence was obtained however, that the cat, dog, and rabbit erythrocyte enzymes were capable of hydrolysing these aliphatic esters. MENDEL AND RUDNEY<sup>11</sup> divided cholinesterases into two main types which they termed 'true' and 'pseudo' respectively. 'True' cholinesterases, which occur in the brain and erythrocytes of many species, were stated to be active only against acetyl choline and closely related compounds, while the 'pseudo' enzymes, *e.g.*, from horse serum and dog pancreas, are capable of hydrolysing both choline and non-choline esters. It was further stated<sup>10</sup> that 'pseudo' cholinesterases hydrolyse benzoyl choline but not acetyl- $\beta$ -methyl choline, while the reverse holds for the 'true' enzymes. Cat, dog, and rabbit erythrocytes were included in the 'true' class on the basis of their ability to hydrolyse acetyl- $\beta$ -methyl choline and failure to attack benzoyl choline, despite the data presented by RICHTER AND CROFT. NACHMANSOHN AND ROTHENBERG<sup>13</sup> suggested that cholinesterases may be divided into 'specific' and 'non specific' types which seem approximately to correspond with MENDEL'S 'true' and 'pseudo' classes.

However, apart from the anomaly referred to above, as far as non-choline esters are concerned these conclusions appear to have been based almost exclusively on the failure of certain arbitrarily selected 'true' or 'specific' cholinesterases to hydrolyse tributyrin and methyl butyrate.

BODANSKY<sup>6</sup> claimed that human erythrocyte cholinesterase preparations, freed from the accompanying ali-esterase by the method of MENDEL AND RUDNEY<sup>11</sup> would nevertheless rapidly hydrolyse triacetin. The mouse brain enzyme was also shown to attack this substrate.

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The specificity data already published on the human erythrocyte cholinesterase is summarized in Table I.

TABLE I  
SPECIFICITY OF THE HUMAN ERYTHROCYTE CHOLINESTERASE

Compound	Investigator
A. Compounds hydrolysed	
Acetyl choline	
Acetyl- $\beta$ -methyl choline . . . . .	4, 10
Acetyl- $\alpha$ -methyl choline . . . . .	
Acetyl erythro- $\alpha$ $\beta$ -dimethyl choline . .	4
Acetyl threo- $\alpha$ $\beta$ -dimethyl choline . . .	
Glyceryl triacetate . . . . .	6
Propionyl choline . . . . .	13
B. Compounds not hydrolysed or hydrolysed only at a very slow rate	
Benzoyl choline . . . . .	10
Butyryl choline . . . . .	13
Carbamyl choline . . . . .	
Tributyrin . . . . .	11, 14
Methyl butyrate . . . . .	

Part A of Table I shows that all the compounds so far known to be hydrolysed in the presence of the erythrocyte enzyme are acetates, with the exception of propionyl choline, which is however hydrolysed much less rapidly than the corresponding acetate. No acetates appear in Part B. These data seemed to indicate that the presence or absence of an acetyl group might be of importance in determining whether or not a given substrate is hydrolysed. Consequently a large number of substrates, mainly acetyl esters, have been tested against erythrocyte preparations which have been partially purified to free the cholinesterase from the known accompanying ali-esterase. The majority of the compounds tested are in fact hydrolysed, although in general not at very high rates.

A preliminary report of this work has already been given<sup>3</sup>.

#### EXPERIMENTAL

##### *Source of enzyme*

Fresh blood was kindly provided by the E.M.S. blood transfusion service. The cells were separated by centrifuging, washed twice with 0.9% saline and the cholinesterase partially purified by a modification of the method of MENDEL AND RUDNEY<sup>11</sup>. The method described by these workers did not in the author's hands lead to preparations completely freed from ali-esterase activity present in the unpurified material.

The cells from one pint of blood are laked in glass distilled water (500 ml) and shaken with acid washed kieselguhr (65 g). This mixture is filtered through a large Buchner and the solid washed as described by MENDEL AND RUDNEY. After the cake has become almost dry, it is shaken with 300 ml of 0.15% sodium bicarbonate and centrifuged for ten minutes at 2000 r.p.m. The turbid supernatant is removed from the kieselguhr, centrifuged at high speed for two hours, and the resulting clear supernatant discarded.

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The precipitate is suspended in 0.2% bicarbonate, centrifuged once more and finally suspended in 40 ml of 0.2% bicarbonate, giving a turbid, almost colourless, liquid highly active against acetyl choline.

*Substrates* (see footnote)

*Choline esters.* Acetyl choline chloride (ACh), butyryl choline chloride (BuCh), acetyl- $\beta$ -methyl choline chloride (MCh), benzoyl choline bromide (BCh)\*\*\*.

*Non-choline esters*

Formates: n-Butyl (BuFo), prim. iso-amyl (isoAmFo).

Acetates: Ethyl (EtAc), n-propyl (PrAc), n-butyl (BuAc), iso-butyl (isoBuAc), n-amyl (AmAc), prim. iso-amyl (isoAmAc), n-hexyl (HxAc)\*, benzyl (BzAc), 1:3-dimethyl butyl (1:3 DiMeBuAc), 2-ethyl butyl (2EtBuAc), 2-ethyl hexyl (2EtHxAc), mono and triacetin (MA, TA), 3:3-dimethyl butyl (3:3DiMeBuAc)\*\*.

Propionates: Ethyl (EtPr), n-propyl (PrPr)\*, n-butyl (BuPr)\*, n-amyl (AmPr), prim. iso-amyl (isoAmPr).

n-Butyrates: Methyl (MeBu), ethyl (EtBu), n-propyl (PrBu), n-butyl (BuBu), n-amyl (AmBu), prim. iso-amyl (isoAmBu), tributyrin (TB).

*Inhibitors*

1.  $\beta\beta'$ -dichlorodiethyl-N-methylamine hydrochloride (DDM).
2. Di-isopropylfluorophosphonate (DFP). (Kindly provided by Dr B. C. SAUNDERS).

*Estimation of enzymic activity*

Esterase activity was determined manometrically, using AMMON'S<sup>5</sup> adaptation of the Warburg technique. (For details see ADAMS AND THOMPSON<sup>1</sup>). In the inhibitor experiments it was found essential to control carefully the time during which the inhibitor and enzyme are incubated in the absence of substrate, in order to obtain reproducible results. This is true of both DDM and DFP (vide MACKWORTH AND WEBB<sup>9</sup> and NACHMANSOHN *et al.*<sup>12</sup> on DFP). As in the previous paper<sup>1</sup> 15 minutes have been allowed. All measurements have been made in  $\mu$ l CO<sub>2</sub>/30 minutes (readings taken over the 5-35 minute period).

In the normal procedure followed with the non-choline esters, a sufficient quantity to give a final concentration of approximately 0.1 Molar was pipetted into the side bulbs of the WARBURG vessels, and covered with 0.5 ml of bicarbonate buffer. However, the use of certain esters under these conditions resulted in the occurrence of negative readings during the experiment, particularly on the non-enzymic control manometers, which were not reproducible and gave poor duplicates. The effect was most marked with iso BuAc, EtBu, and EtPr, and was shown to some extent by MeBu, PrPr, PrAc, and BuAc. These esters are all soluble in water to the extent of *ca.* 0.5-2%, and the negative readings were ascribed to a slow solution of ester in the aqueous phase, and contamination

Unmarked = commercial samples.

\* Prepared in the laboratory. (n-Hexyl acetate from n-hexyl alcohol and acetic anhydride, n-propyl and n-butyl propionates from the alcohols heated with propionic acid in the presence of 3% sulphuric acid).

\*\* Kindly provided by Dr A. J. BIRCH.

\*\*\* Kindly provided by the Govt. Experimental Station, Porton, England.

Pure primary iso-amyl compounds could not be obtained, nor any primary-iso-amyl alcohol from which to prepare them. Consequently, ordinary 'amyl' esters of commercial origin have been employed as substitutes. These contain *ca.* 90% of primary iso-amyl ester.

All volatile esters were redistilled before use, in most cases after washing with sodium bicarbonate solution. The non-choline esters have been employed throughout at 0.1 Molar concentration and BuCh, MCh, and BCh at 0.03 M.

*References p. 14.*



of undissolved ester with water, either or both of these processes resulting in a lowering of vapour pressure. Shaking these compounds with the appropriate quantity of bicarbonate buffer at 40° C during 3 hours, followed by a rapid pipetting of the resulting emulsion into the main compartments of the WARBURG vessels (enzyme in the side bulb) served to minimize the effect and reasonably good duplicates were obtained with this modified procedure.

With the more volatile compounds, the  $N_2 + 5\% CO_2$  gas mixture was passed very slowly through the vessels in an effort to prevent undue loss by evaporation.

## RESULTS

### *Evidence for the homogeneity of the enzyme preparations*

The question whether the hydrolysis of a number of esters by a tissue preparation is due to a single enzyme can only finally be settled by the isolation and examination of the pure hydrolytic agent. Such procedures usually involve considerable technical difficulties, and, in fact, no pure erythrocyte cholinesterase has ever been prepared. Consequently this problem has been attacked by a combination of two of the classical methods available for the investigation of impure preparations. These are:

1. Summation experiments with mixed esters. It may be shown on classical MICHAELIS theory that if a single enzyme is responsible for the hydrolysis of two different esters, the rate at which a mixture of the two is hydrolysed will lie between the rates of hydrolysis of each separately. On the other hand if two distinct enzymes are present, each hydrolysing one ester, the mixture will be hydrolysed at a rate which is the arithmetical sum of the rates of hydrolysis of each separately. This is of course only true if the two systems are independent, for example, that neither substrate inhibits the other's enzyme.

2. Since no single method can give a completely unequivocal answer inhibitor studies have also been carried out. To strengthen the evidence two different inhibitors have been employed, and the results expressed as the ratio, for each given substrate, of the concentrations of the individual inhibitors required to produce 50% inhibition of enzymic activity (See <sup>1, 2</sup> for details of the method).

### *Summation experiments*

MCh rather than ACh has been employed as a standard in those summation experiments which include a choline ester, for the following reasons.

- a. MCh exhibits a classical MICHAELIS activity- substrate concentration curve<sup>10</sup> (and confirmed in this laboratory), and is consequently far less sensitive to small variations in substrate concentration than ACh with its humped (MURRAY-HALDANE) type of curve.

- b. It is not clear that a straightforward answer would necessarily be expected to result from the exposure to the enzyme of a mixture of an insoluble or partly soluble non-choline ester and a soluble substrate showing this more complicated type of kinetic behaviour.

- c. The lower rate at which MCh is hydrolysed renders it a more convenient substrate. The results of the summation experiments appear in Table II.

In Group I MCh is taken in conjunction with various acetates. The individual experiments run across the columns. Column (a) expresses the rate of hydrolysis of MCh, column (b) the rate of hydrolysis by the same quantity of enzyme of the acetate ester under investigation, and column (c) the corresponding rate of hydrolysis of a mixture of the two compounds. Although from experiment to experiment the same quantity of enzyme has usually been taken, this practice has not been followed in

variably. Column (d) shows the sum [(a) + (b)] of the rates of hydrolysis of the two esters individually, and it will be observed that in no case does the observed rate (c) approach this figure. In fact, in practically every case the observed rate for the mixture lies between the rates for the individual esters, in satisfactory agreement with the supposition that a single enzyme is responsible for the observed hydrolysis. This result is expressed in column (e) which gives the algebraic difference between the 'mixed ester' rate and the rate of hydrolysis of the more rapidly hydrolysed substrate. In one or two cases the mixed esters are hydrolysed slightly more rapidly than either separately, but the effect is hardly significant.

TABLE II  
SUMMATION EXPERIMENTS

Group I. Acetyl- $\beta$ -methyl choline + non-choline acetate esters. (Activities in $\mu\text{l CO}_2/30 \text{ min}$ )					
	(a) MCh	(b) Non choline esters	(c) Mixed esters	(d) (a) + (b)	(e) (a) - (c)
MCh + n-propyl acetate . .	238 241 221	64 71 64	223 225 212	302 312 285	—15 —16 —9
MCh + n-butyl acetate . .	235 240 232	119 113 118	233 237 228	354 353 352	—2 —3 —4
MCh + n-amyl acetate . .	244 255 241	107 118 103	238 253 226	351 375 344	—6 —2 —15
MCh + iso-amyl acetate . .	262 177 250	179 132 180	263 175 247	441 319 430	+1 —2 —3
MCh + benzyl acetate . . .	163 245 250	101 167 181	159 246 254	264 412 431	—4 +1 +4
MCh + triacetin . . . . .	189 188 150	235 235 180	183 190 146	426 425 330	—6 +2 —4
MCh + monoacetin . . . .	232 237 245	136 141 145	229 221 230	368 378 390	—3 —16 —15
Group II. Acetyl- $\beta$ -methyl choline + non-choline propionate esters. (Activities in $\mu\text{l CO}_2/30 \text{ min}$ )					
	(a) MCh	(b) Non choline esters	(c) Mixed esters	(d) (a) + (b)	(e) (a) - (c)
MCh + n-amyl propionate .	241 242 232	15 13 13	152 138 139	256 255 255	—89 —104 —93
MCh + iso-amyl propionate	234 240 233	69 63 74	210 212 204	303 303 307	—24 —28 —29

TABLE II (Continued)

Group III. Ethyl acetate + propionate and butyrate esters. (Activities in $\mu\text{l CO}_2/30 \text{ min}$ )					
	(a) Ethyl acetate	(b) Other esters	(c) Mixed esters	(d) (a) + (b)	(e) (a) or (b) - (c)
EtAc + n-amyl propionate	43	28	37	70	— 6
	41	29	37	71	— 4
EtAc + iso-amyl propionate	27	116	111	143	— 5
	33	123	101	156	—22
	33	127	115	160	—12
EtAc + tributyrin . . . .	56	21	56	77	0
	51	22	49	73	— 2
	47	22	46	69	— 1
EtAc + iso-amyl butyrate .	50	19	51	69	+ 1
	47	20	48	67	+ 1

Group II presents the results of summation experiments with MCh + AmPr and isoAmPr, in which, again, no positive summation is observed.

Laked erythrocytes treated according to the original method of MENDEL AND RUDNEY tended to show a small degree of positive summation which appeared most marked in the cases of iso AmAc and BzAc (up to 10 %). These preparations were also slightly active against tributyrin, the rate of hydrolysis being 3-4 % of the MCh rate. The modified purification procedure already described eliminated the small partial summation but the TB rate was only reduced to 2-2.5 % of the MCh rate. An active ali-esterase which readily attacks tributyrin accompanies the cholinesterase is unpurified material, and MENDEL AND RUDNEY<sup>11</sup> claim that its elimination reduced the TB hydrolysis to zero. Evidence will be presented however, that the cholinesterase is capable of hydrolysing TB very slowly, and as a practical test of freedom from ali-esterase no preparations have been used which did not comply with the following conditions:

- Showing no partial positive summation with MCh and iso AmAc.
- Having a TB activity less than 2.5 % of the MCh activity.

In the third group (Table II) ethyl acetate has been substituted for MCh. It is first shown that no positive summation occurs with AmPr and isoAmPr, which themselves give no positive summation with MCh, and then that there is also no, or very little, positive summation with the low TB and isoAmBu activities. The suitability of EtAc as a subsidiary standard is also borne out by the fact that the activity of unpurified erythrocytes towards this substrate is only *ca.* 15% greater than the activity with purified preparations, indicating that EtAc is only slowly attacked by the ali-esterase occurring in unpurified material.

The results with triacetin appear somewhat anomalous in that although this substrate is hydrolysed more rapidly than MCh the rate of hydrolysis of the MCh + TA mixture is, if anything, slightly below the MCh rate. In all the other summation experiments MCh has been the more rapidly split substrate.

#### *Inhibition experiments*

The inhibitory effects of various concentrations of DDM and DFP on the enzymic hydrolysis of ACh, MCh, and the two representative non-choline esters TA and AmAc were determined with a view to providing further evidence for the homogeneity of the enzyme preparations. From the inhibitor results the DDM/DFP  $I_{50}$  ratios<sup>1</sup> have been

calculated using the statistical method developed by ADAMS AND WHITTAKER<sup>2</sup> in connection with the plasma enzyme.

Straight lines result on plotting the reciprocal of the inhibitor concentrations against the reciprocal of the % inhibitions (Figs 1 and 2), a necessary condition for the application of the statistical method. In Figs 1 and 2, the lines drawn through the points are

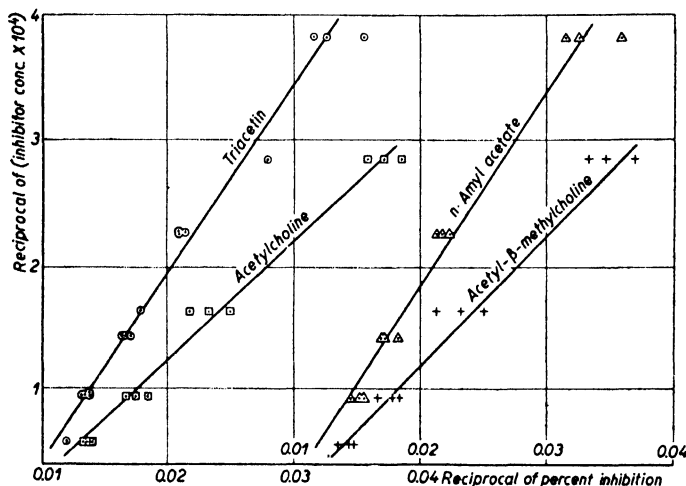


Fig. 1. Inhibition of purified erythrocyte cholinesterase by DDM. The straight lines are the regression lines of ordinates on abscissae. Upper abscissa scale: n-amyl acetate, acetyl- $\beta$ -methyl choline; lower scale, triacetin, acetyl choline.

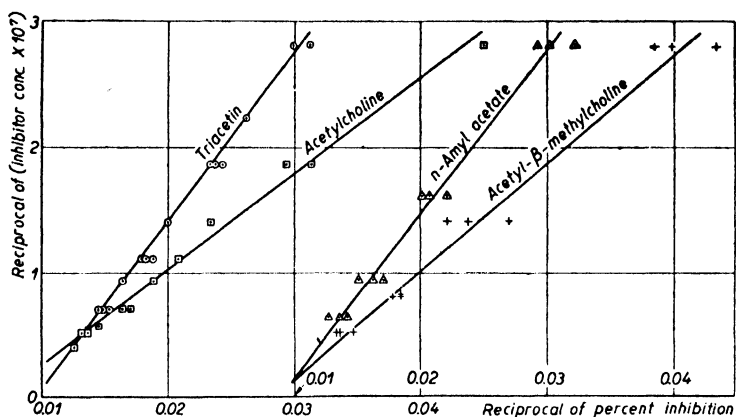


Fig. 2. Inhibition of purified erythrocyte cholinesterase by DFP. The straight lines are the regression lines of ordinates on abscissae; abscissa scales as in Fig. 1.

the lines of closest fit calculated from the data. The most probable values and the upper and lower limits of the  $I_{50}$  concentrations for the individual inhibitors are shown in Table III and the corresponding  $I_{50}$  ratios in Table IV.

Although there is a greater variation in the  $I_{50}$  ratios than might have been expected from the results obtained with the plasma enzyme<sup>2</sup>, they formally establish the identity of the enzymes hydrolysing ACh and MCh, and provide further evidence to show that the non-choline esters are also being hydrolysed by one and the same enzyme.

References p. 14.

TABLE III  
 $I_{50}$  VALUES FOR DDM AND DFP  
 ( $Y_{50}$  values, DDM ( $M^{-1} \cdot 10^4$ ), DFP ( $M^{-1} \cdot 10^7$ ). ( $Y_{50} = 1/I_{50}$ )

Substrate	Number of observations	Y <sub>50</sub>	Standard error(s)	Range of Y <sub>50</sub> for which P = 0.2		I <sub>50</sub> values and limits outside which less than 20 % of estimates would be expected to fall		
				Y <sub>50</sub> + ts	Y <sub>50</sub> — ts	upper	most probable	lower
DDM								
ACh	12	12.4	0.33	12.9	11.9	8.4	8.05	7.75
MCh	12	12.0	0.45	12.6	11.4	8.75	8.35	7.95
AmAc	12	18.7	0.69	19.7	17.7	5.6	5.35	5.0
TA	16	19.2	0.43	19.8	18.6	5.4	5.2 (· 10 <sup>-6</sup> Molar)	5.05
DFP								
ACh	12	10.1	0.44	10.7	9.5	10.5	9.9	9.35
MCh	12	9.8	0.45	10.4	9.2	10.9	10.2	9.6
AmAc	12	14.4	0.42	15.0	13.8	7.25	6.95	6.65
TA	14	13.8	0.35	14.3	13.3	7.5	7.25 (· 10 <sup>-8</sup> Molar)	7.0

TABLE IV  
 $I_{50}$  (DDM/DFP) RATIOS FOR ACh, MCh, AmAc, AND TA

Substrate	Most probable values of $I_{50}$ ratios and limits outside which less than 2 % of estimates would be expected to fall.		
	Upper limit	Most probable value	Lower limit
ACh	900	815	740
MCh	910	820	730
AmAc	845	770	705
TA	770	720	670

The  $I_{50}$  ratio variations are considerably smaller than the range of values for the individual inhibitors.

#### *Rate of hydrolysis in relation to substrate configuration*

Having demonstrated by summation and inhibition experiments that a single enzyme is responsible for the observed hydrolysis, the preparations were tested against a number of other substrates in an attempt to map out the approximate specificity pattern of the enzyme, and also to establish any possible relationships between substrate molecular configuration and rate of hydrolysis. The collected results are given in Table V.

Several generalization are possible from the data, and these will be considered in detail. The dependence of the rate of hydrolysis on the size of the alkyl group is illustrated in Fig. 3 which shows the rate plotted against the number of C atoms in the n-alkyl chain for a number of acetates, propionates, and butyrates. It will be observed that although the propionates are hydrolysed much less rapidly than the corresponding

TABLE V

RATES OF HYDROLYSIS OF CHOLINE AND NON-CHOLINE ESTERS IN THE PRESENCE OF PURIFIED ERYTHROCYTE CHOLINESTERASE, EXPRESSED AS PERCENTAGES OF THE RATE OF HYDROLYSIS OF ACETYL- $\beta$ -METHYL CHOLINE. ALL SUBSTRATES AT 0.1 MOLAR CONCN. UNLESS OTHERWISE STATED

Substrate	Rate of hydrolysis	Substrate	Rate of hydrolysis
ACETYL- $\beta$ -METHYL CHOLINE (0.03 M) .	100	3 : 3-Dimethyl butyl acetate . .	180
Acetyl choline (0.006 M) . . . . .	200	Benzyl acetate . . . . .	68
Acetyl choline (? M) (estimated peak)	ca. 300	Ethyl acetoacetate . . . . .	6
Benzoyl choline (0.03 M) . . . . .	1.5	Monoacetin . . . . .	58
Butyryl choline (0.03 M) . . . . .	5	Triacetin . . . . .	125
Ethyl acetate . . . . .	6.2	Ethyl propionate . . . . .	2
n-Propyl acetate . . . . .	29	n-Propyl propionate . . . . .	9
n-Butyl acetate . . . . .	49	n-Butyl propionate . . . . .	16
n-Amyl acetate . . . . .	45	n-Amyl propionate . . . . .	5.7
n-Hexyl acetate . . . . .	22	iso-Amyl propionate . . . . .	29
iso-Butyl acetate . . . . .	20	n-Butyl formate . . . . .	10
iso-Amyl acetate . . . . .	72	iso-Amyl formate . . . . .	10
1 : 3 Dimethyl butyl acetate . . . . .	26	iso-Amyl butyrate . . . . .	ca. 2
2-Ethyl butyl acetate . . . . .	65	Tributyryn . . . . .	ca. 2
2-Ethyl hexyl acetate . . . . .	23		

No hydrolysis could be detected with the n-alkyl butyrates from methyl to amyl.

acetates, the maximum in both cases occurs with the n-butyl ester. Fig. 4 illustrates the effect of acyl group size for two groups of fatty acid esters, the n-butyl and iso-amyl series from formate to butyrate. The sharp peak at the acetate point is evident, the propionates being hydrolysed relatively slowly and the butyrates and formates very slowly or not at all.

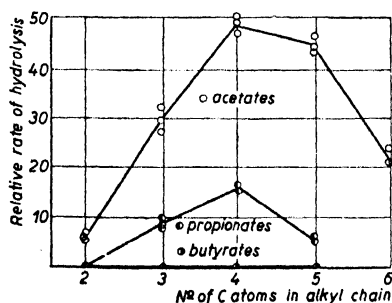


Fig. 3. Effect of alkyl chain length on rate of hydrolysis of n-alkyl esters in presence of erythrocyte cholinesterase. Rate of hydrolysis is expressed as a percentage of rate of hydrolysis of acetyl- $\beta$ -methyl choline.

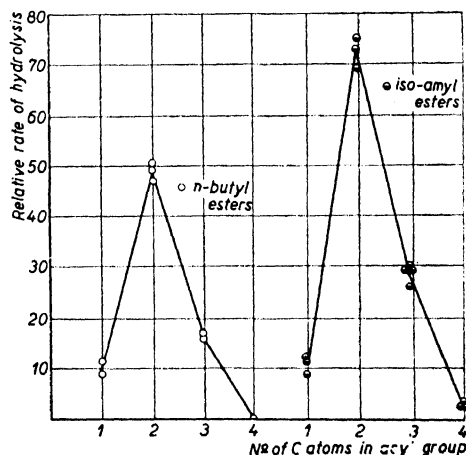


Fig. 4. Effect of acyl chain length on rate of hydrolysis of two series of aliphatic esters in presence of erythrocyte cholinesterase. Rate of hydrolysis is expressed as a percentage of rate of hydrolysis of acetyl- $\beta$ -methyl choline.

It has already been pointed out that n-butyl acetate is the most rapidly hydrolysed straight chain ester, but Table V shows that it is split at only half the rate of MCh,

and something like 1/6 of the estimated peak rate of ACh. Extra carbon atoms attached to, or carbon atoms removed from, the end of the alkyl chain lead only to a reduction in the rate. However, extra carbon atoms accommodated as branches may result in a substantial increase. This is illustrated in Table VI which shows the alkyl carbon skeletons of a number of branched chain acetates and the corresponding rates of hydrolysis.

TABLE VI

ERYTHROCYTE CHOLINESTERASE. RELATIONSHIP BETWEEN SUBSTRATE CONFIGURATION AND RATE OF HYDROLYSIS

(Rates of hydrolysis expressed as percentages of the MCh rate)

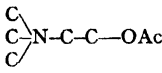
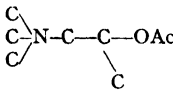
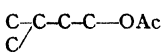
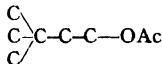
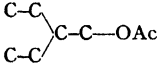
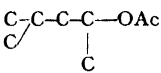
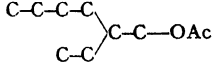
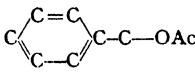
Acetyl choline  <i>ca.</i> 300	Acetyl- $\beta$ -methyl choline  100	.
n-Butyl acetate $C-C-C-C-OAc$ 49	iso-Amyl acetate  72	3 : 3-Dimethyl butyl acetate  180
2-Ethyl butyl acetate  65	1 : 3-Dimethyl butyl acetate  26	2-Ethyl hexyl acetate  23
Benzyl acetate  68		

Table VI indicates that branching at the end of the chain, as in iso-amyl acetate, increases the rate of hydrolysis, and this increase is maintained in 2 EtBuAc where the branching occurs at the 2 position. Benzyl acetate, which may be regarded as being derived from the last compound by ring closure and conjugation is also rapidly attacked. The increase from n-BuAc to isoAmAc, particularly, appeared to indicate the possibility that the more closely the configuration of a given substrate approaches that of acetyl choline the more rapidly it is hydrolysed. In order to test this hypothesis a small quantity of 3 : 3-dimethyl butyl acetate, which may be regarded as the carbon analogue of acetyl choline, was obtained and found to be hydrolysed at approximately 180% of the MCh rate, *i.e.*, more rapidly than any other ester with the exception of ACh itself.

## DISCUSSION

The evidence from summation and inhibition experiments appears to establish beyond reasonable doubt the homogeneity of the enzyme preparations. As has already been pointed out, the  $I_{50}$  ratios have not been so closely similar as might have appeared possible from the results of ADAMS AND WHITTAKER<sup>2</sup>, but the values for the choline esters are practically identical, and the most probable value of the ratio for the choline esters and for *n*-amyl acetate are well within each other's ranges. Triacetin falls outside the choline ester values but just inside the AmAc range. Compared with some single inhibitor results which have appeared in the literature as evidence for the homogeneity of enzyme preparations, the agreement between the ratios may be regarded as excellent. Possible reasons for the divergence may be put forward on the basis of SINGER's<sup>15</sup> observations on the inhibitions of a lipase by various non-competitive inhibitors. This author showed that variations in inhibition by a given concentration of a non-competitive inhibitor in the presence of a number of substrates could be correlated with variations in the molecular size of the substrate. DFP certainly inhibits in a manner which is almost entirely non-competitive, and although DDM has on the basis of THOMPSON's<sup>18</sup> earlier work hitherto been classed as competitive there now appears to be considerable doubt regarding this point. The inhibitor appears to resemble DFP in some respects *e.g.*, the degree of inhibition obtained increases considerably with the time of incubation. The variations in inhibition observed using DDM or DFP alone cannot readily be explained in this case on the basis of differences in the molecular size of the substrates, but differences in molecular shape, and the absence of an integral positive charge from the non-choline esters may well act in some manner analogous to that suggested by SINGER. An exact agreement between the  $I_{50}$  ratios using arbitrarily chosen inhibitors would therefore be unlikely, and could only be obtained if the relative effects on the individual inhibitors were identical. Nevertheless, it might be expected that the use of the ratio would give a closer agreement between a number of substrates, and that this is true for the system under consideration may be seen by a comparison between the individual inhibitor results in Table III and the ratios in Table IV.

The results confirm the hypothesis that the acetyl group plays a major role in the configuration of rapidly hydrolysed substrates. That the approach of the alcohol group to the choline structure is also of importance seems proven by the very high rate at which the carbon analogue of acetyl choline, 3 : 3-dimethyl butyl acetate, is hydrolysed. This compound is also of interest in that its molecular configuration, shape (including bond lengths) and, consequently, the strength and distribution of the VAN DER WAALS forces holding it in contact with the enzyme, should all be practically identical with the corresponding properties of acetyl choline. It is reasonable to suppose therefore that any differences in the action of the enzyme on the two substrates may be ascribed to the positive charge carried by ACh.

The similar rates at which 2 EtBuAc and isoAmAc are hydrolysed appear to indicate that branching at the 2 position is only slightly less favourable than branching at the end of the chain. Unfortunately however, no 2 : 2-diethyl butyl acetate has been available for comparison with 3 : 3-DiMeBuAc. Branching next to the ester link reduces the rate, as in 1 : 3-DiMeBuAc (Table VI) (derived from iso-amyl acetate by the introduction of a methyl group in the 1 position) which is hydrolysed much less rapidly than the parent compound. ACh and MCh are analogous.



It has already been mentioned that the enzyme preparations are slightly active against tributyrin, and that the evidence from summation experiments with EtAc given in Table II indicates that the cholinesterase is responsible. In view of the very low activity it has not been possible to carry out any precise inhibitor experiments, but the use of concentrations of DDM and DFP which would be expected to produce a 50% inhibition of the cholinesterase have shown that the degree of inhibition obtained with both inhibitors has not differed very greatly from this figure. The summation experiments with isoAmBu and EtAc also provide evidence for the capacity of the enzyme to hydrolyse butyrates containing a favourable alcohol group, at a slow rate.

Mono and triacetin do not fit readily into the simple hypothesis that closeness of approach to the ACh structure is the sole factor in determining the rate of hydrolysis. TA is the second most rapidly split non-choline ester and suggests that other as yet unknown steric configurations, more or less unrelated to ACh, may be permissible. The somewhat anomalous behaviour of TA in both the summation and inhibition experiments has already been pointed out, and this would seem to indicate that this substrate is associated with the enzyme in some slightly different manner. However its rapid rate of non-enzymic hydrolysis must be borne in mind in attempting an interpretation of the results.

Since the commencement of this work, results by other authors have appeared which are of interest. BOVET NITTI<sup>7</sup> examined the hydrolytic action of snake venom enzyme preparations against acetyl choline and a number of non-choline esters. Her results show that the preparations hydrolysed acetyl choline and all the non-choline acetate esters investigated. Tributyrin and ethyl propionate were not attacked. However no evidence that the choline and non-choline esters were being hydrolysed by the same enzyme was submitted, and although BOVET NITTI appears to assume that they were, she states that the enzyme responsible is not a cholinesterase and refers to it as an 'acetylase'.

HOLTON<sup>8</sup> has confirmed BODANSKY'S<sup>6</sup> observation on the hydrolysis of triacetin by brain, and has also provided evidence to show that the snake venom cholinesterase is also capable at least of attacking this substrate.

ZELLER<sup>20, 21</sup> has confirmed and extended the work of BOVET NITTI, showing that the choline and non-choline ester hydrolysis is due to the same enzyme, and proposed that it should be classified as a third ('C') type of cholinesterase in addition to the 'E' and 'S' types already distinguished by ZELLER AND BISSEGER<sup>22</sup>.

Despite the wide specificity range of this so-called specific erythrocyte enzyme, the results reported here have demonstrated little or nothing which could be interpreted as evidence that acetyl choline is not, in many respects, a unique substrate. So far as is known at present ACh is hydrolysed at its peak rate nearly twice as rapidly as any other ester, and further, appears to be the only substrate exhibiting a humped (MURRAY-HALDANE) activity-substrate concentration curve, which, since the peak occurs at a relatively low substrate concentration, would particularly adapt this enzyme to the rapid removal of ACh under physiological conditions.

There seems little doubt however that the terms 'true' and 'specific' as originally defined are no longer tenable. The specificity of the brain enzymes has not yet been fully investigated, but there is every reason to believe that the range is much wider than has hitherto been assumed and may well be expected to be as great as that of the erythrocyte enzyme examined here. Neither does there now seem to be any necessity

for the introduction of ZELLER's 'C' type cholinesterase, originally suggested on the basis of the ability of the snake venom enzyme to hydrolyse non-choline esters. However, certain differences appear when the substrate specificity pattern of this enzyme is compared with that of human erythrocytes. For example, according to BOVET NITTI<sup>7</sup>, iso-amyl and n-butyl acetates are hydrolysed only at a very slow rate, and ethyl acetate much more rapidly, a reversal of the results described here.

It is hoped to continue this work by mapping out the specificity pattern of both human plasma and a typical brain enzyme in order to investigate the precise differences in properties which exist between different tissue cholinesterases.

#### *Acknowledgements*

I am much indebted to Dr V. P. WHITTAKER for his generous help and advice. I am also grateful to Dr A. J. BIRCH for the gift of 3 : 3-dimethyl butyl acetate prepared by him, and to Dr H. BLASHKO for drawing my attention to the work of BOVET NITTI. Finally I wish to thank the Department of Scientific and Industrial Research, London, for a grant.

#### SUMMARY

1. The human erythrocyte cholinesterase, hitherto regarded as a highly specific enzyme, has been shown to catalyse the hydrolysis of a large number of non-choline esters.
2. All the acetates investigated have been hydrolysed. The corresponding propionates and formates are attacked less readily, and butyrates very slowly or not at all.
3. In general the more closely the alcohol group simulates the choline configuration, the more rapidly is the ester hydrolysed. Of the substrates investigated, 3 : 3-dimethyl butyl acetate, the carbon analogue of acetylcholine is the most rapidly hydrolysed next to acetylcholine itself.
4. For the differentiation of cholinesterases the terms 'true' and 'pseudo', or 'specific' and 'non-specific', as originally defined, are no longer tenable.

#### RÉSUMÉ

1. La cholinesterase des érythrocytes de l'Homme, qu'on croyait être une enzyme hautement spécifique, catalyse l'hydrolyse d'un grand nombre d'esters non-choliniques.
2. Tous les acétates examinés sont hydrolysés: les propionates et les formates correspondants sont attaqués moins facilement, et les butyrates très lentement ou pas du tout.
3. En général, plus le groupement alcoolique d'un ester se rapproche de la configuration cholinique, plus l'hydrolyse de l'ester est rapide. L'acétate de 3 : 3-diméthyl butyle, l'analogue carbonique de l'acétylcholine, est le plus rapidement hydrolysé à l'exception de l'acétylcholine elle-même.
4. Les termes 'vrai' ou 'pseudo', 'spécifique' ou 'non-spécifique' qui ont été avancés pour distinguer les différentes espèces de cholinestérases ne peuvent plus être retenus.

#### ZUSAMMENFASSUNG

1. Es konnte gezeigt werden, dass die Cholinesterase der menschlichen Erythrocyten, die bis jetzt als ein hochspezifisches Enzym betrachtet wurde, in der Lage ist, die Hydrolyse einer grossen Anzahl von nicht-Cholinestern zu katalysieren.
2. Alle untersuchten Acetate konnten hydrolysiert werden. Die entsprechenden Propionate und Formiate werden weniger angegriffen und Butyrate sehr langsam oder garnicht.
3. Im Allgemeinen kann gesagt werden: je mehr die Alkoholgruppe der Cholinform ähnelt, desto schneller erfolgt die Hydrolyse des Esters. Von den untersuchten Substanzen wurde 3 : 3-Dimethylbutylacetat, das Kohlenstoffanalogue des Acetylcholins am schnellsten hydrolysiert, mit Ausnahme des Acetylcholins selbst.
4. Für die Differenzierung von Cholinesterasen sind die Begriffe 'echt' und 'pseudo' oder 'spezifisch' und 'unspezifisch' wie ursprünglich definiert, nicht mehr länger haltbar.

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## ELECTRON MICROGRAPHS OF PLANT FIBERS

by

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## INTRODUCTION

The electron microscope permits a much more detailed study of the fine structure of plant fibers than could hitherto be made with the aid of polarization microscope and the methods of X-ray diffraction. As a result, more than eighty years after NÄGELI<sup>1</sup> published his important micellar theory (1858), it has now become possible to visualize the elementary particles he postulated.

( Electron micrographs (RUSKA AND KRETSCHMER)<sup>2</sup> of plant fibers were first published eight years ago, but these early photographs yielded little information that was new about the size, form and systematic arrangement of these submicroscopic particles. Neither has the work done since answered in conclusive fashion questions concerning the development of fibrils of a size near the resolution of the light microscope. These disappointing results should not, however, be laid to the microscope itself, but rather, to inadequate methods of specimen preparation. The commonly employed methods of splitting cellulose structures, such as crushing in a ball mill, splitting with ultrasonic waves or disintegration with chemicals have damaged the natural texture to such an extent that new results concerning the construction of the cell wall have not been apparent in the electron micrographs. We have, in large measure, evaded these difficulties and obtained photographs of unaltered cell wall structures by using a simple, new method of dissection and by metal shadowing the specimen preparations before microscopy. )

## EXPERIMENTAL

( A Waring Blender has been used to prepare satisfactory fiber sections. To do this the fibrous material was first cut to about one cm lengths, suspended in distilled water and then stirred in the blender for five minutes. Stirring of the liquid by the blades of the blender rotating at very high speed tears the suspended fibers to a loose pulp.

) The shreaded fibers were next washed in distilled water and the heavier particles eliminated by sedimentation in a glass cylinder. One drop of finely dispersed material from the supernatant was finally dried on the usual collodion-covered grid and shadowed with either chromium or palladium. )

## RESULTS

Optical microscopic and polarization-optical observations have shown that plant cell walls consist of two layers that differ in their structure. The primary wall is deposited

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on the middle lamella, which consists mainly of pectin and provides the cementing substance between individual cells. This wall is formed during the growth of cells and its thickness amounts to only ca  $0.5 \mu$ . As soon as the cell has reached its final state of growth, deposition of the secondary wall sets in and continues until the cell lumen is almost completely filled.

Under ordinary circumstances the fine structure of these walls cannot be seen with the light microscope, but after staining with Congo red the primary wall appears in the polarizing microscope to be a criss-cross fibrous structure. FREY-WYSSLING<sup>4</sup> has used the polarizing microscope to demonstrate the arrangement of the cellulose in the secondary wall. He has found that in contrast to the primary wall, the secondary wall has its fibrils arranged in parallel layers. The cross-sectional fiber structure he postulates is shown in Fig. 1. According to this scheme the fibrils could either be completely separate, as shown in Fig. 1 (A), or they could merge into neighbours (Fig. 1 (B)), and it

has hitherto been impossible with the indirect methods available to decide between these alternatives.

The present electron micrographs throw light on this question and reveal fibrils having a fairly constant diameter of 250–400 Å. A detailed discussion of the structure of the fibrils will, however, be deferred to a later paper to be published on this subject.

( If fibers are swelled with zinc chloride for optical examination, the secondary walls clearly show in cross section an arrangement of layers.

With a cellulose dye, successive

layers are differently coloured and the double refraction also varies between successive lamellae. Tests made on growing cotton fibers showed that each lamella reflects the daily deposition of cellulose, the thickness of these newly formed fiber layers varying between 0.1 and  $0.5 \mu$ , according to external conditions. )

We propose to investigate, with the help of the following photographs, to what extent the conceptions prevailing today correspond to actual facts.

### Ramie

Though ramie is not one of the important textile fibers of commerce, the excellent arrangement of its cellulose and the absence of large quantities of non-cellulose substances have made it an especially desirable material for scientific studies.

Its two wall types mentioned above, *i.e.*, primary wall (Fig. 2) and secondary wall (Fig. 3), can evidently be clearly distinguished in electron micrographs. In both layers we find the cellulose developed as completely individualized fibrils, having a thickness of about 250 Å. The arrangement of these fibrils is, however, entirely different in the two layers. As indicated by the earlier polarization-optical and X-ray investigations, the secondary wall consists of parallel layers in which the fibrils are in such close contact that it is often hard to distinguish them from one another. The fibrils of the primary

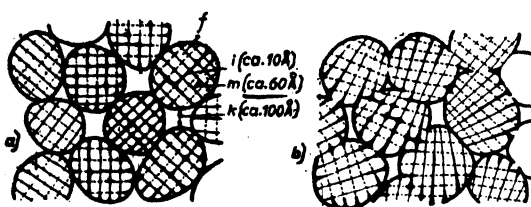


Fig. 1. Schematic cross section of the submicroscopic fiber construction according to FREY-WYSSLING. *f* = submicroscopic microfibrils interspersed with intermicellar spaces (*f*); *m* = schematic sketch of undistorted micelles projected into a plane; *k* = submicroscopic capillaries between microfibrils. In Fig. 1 (A) the microfibrils are completely separate. In Fig. 1 (B) the structure of the microfibrils merge into neighbouring fibrils. (According to FREY-WYSSLING, *Protoplasma*, 27 (1937) 372–411).

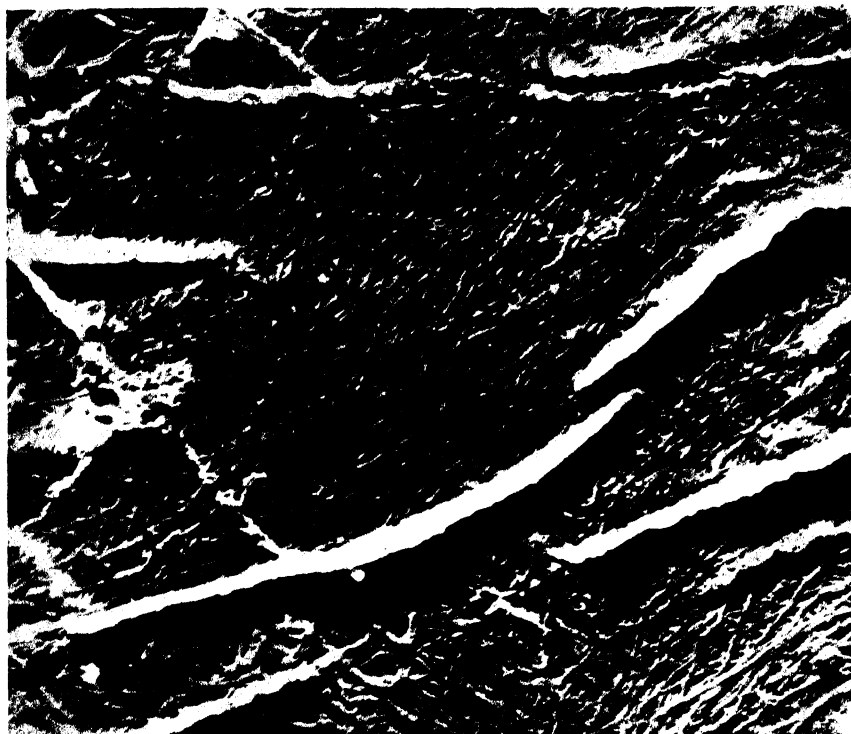
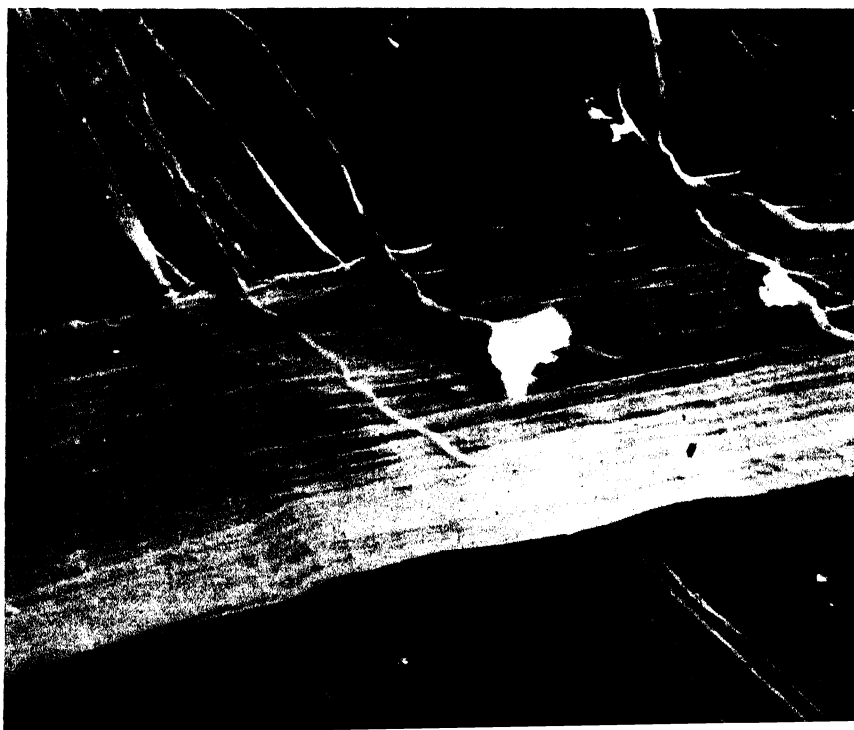


Fig. 2. An electron micrograph of the primary wall of a ramie fiber. Adhering non-cellulosic substances give to the fibers of this and certain other photographs a somewhat diffuse appearance. Magnification = 20 000  $\times$ .



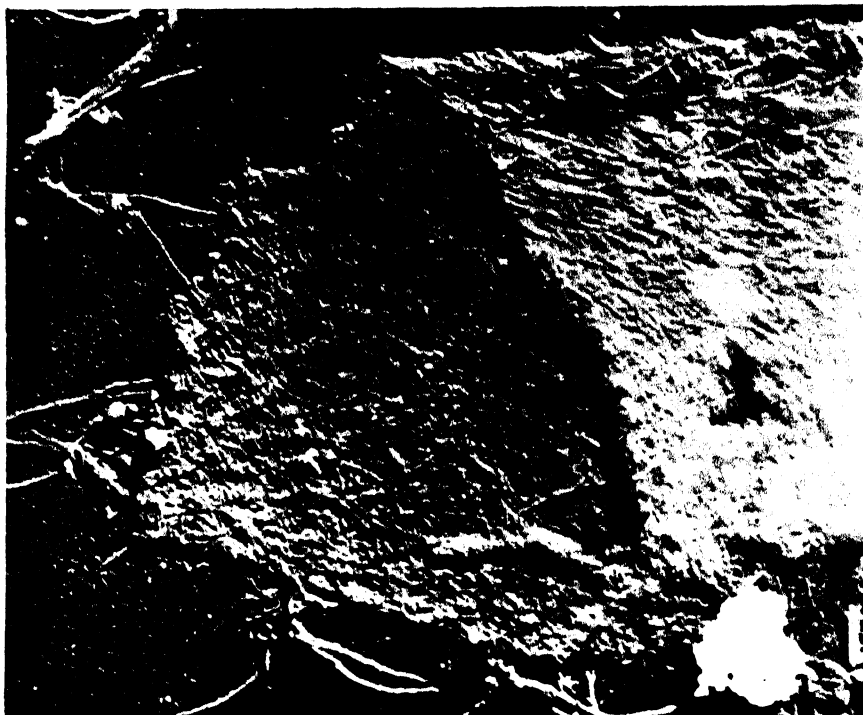


Fig. 4. An electron micrograph of the primary wall of a cotton fiber. Magnification = 20000  $\times$ .



Fig. 5. An electron micrograph of the secondary wall of a cotton fiber. Magnification = 20000  $\times$ .

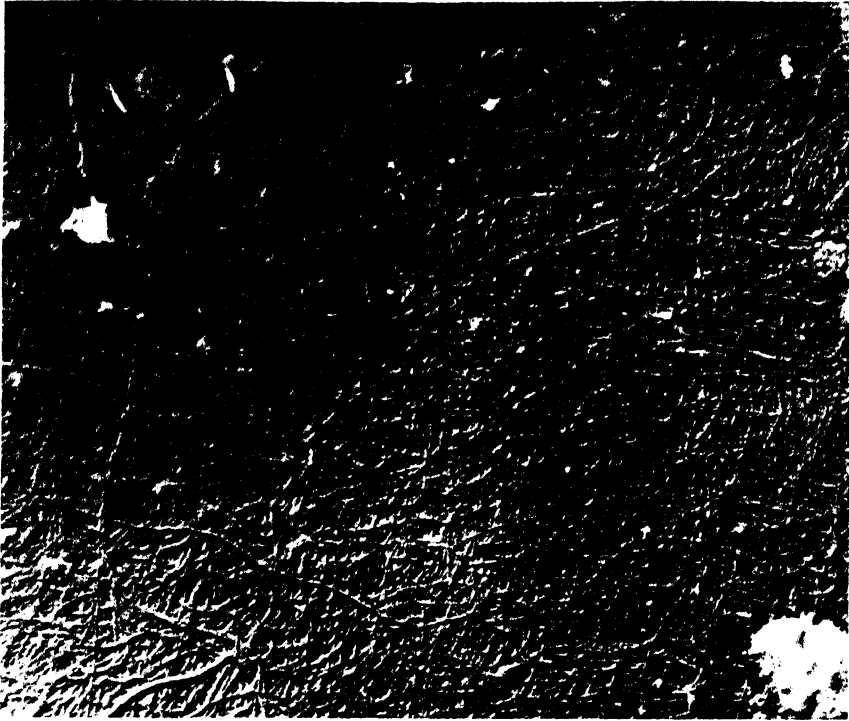


Fig. 6. An electron micrograph of the primary wall of a flax fiber. Magnification = 20 000  $\times$ .

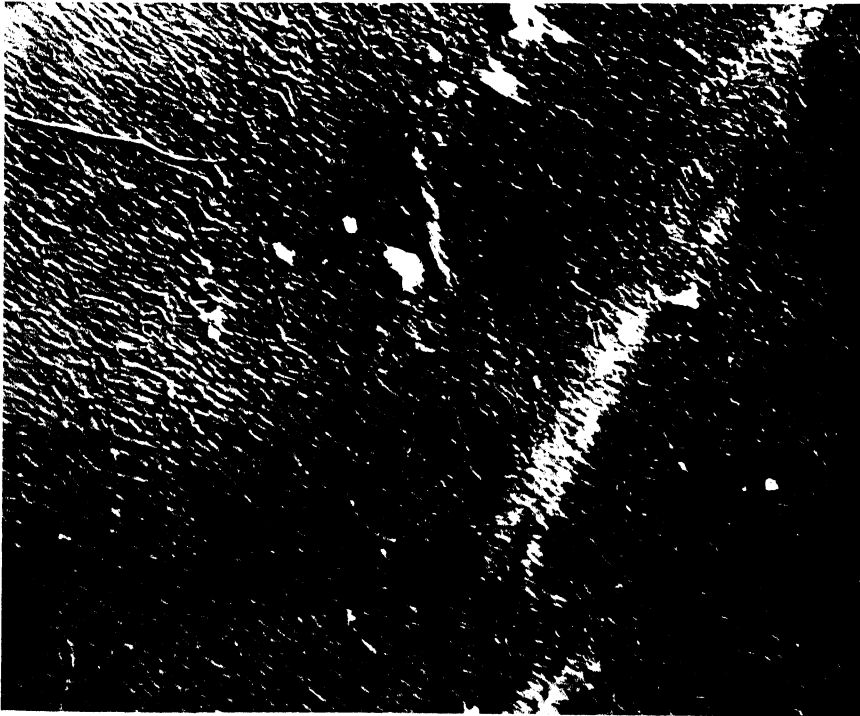


Fig. 7. An electron micrograph of the secondary wall of a flax fibre. Magnification = 20 000  $\times$ .



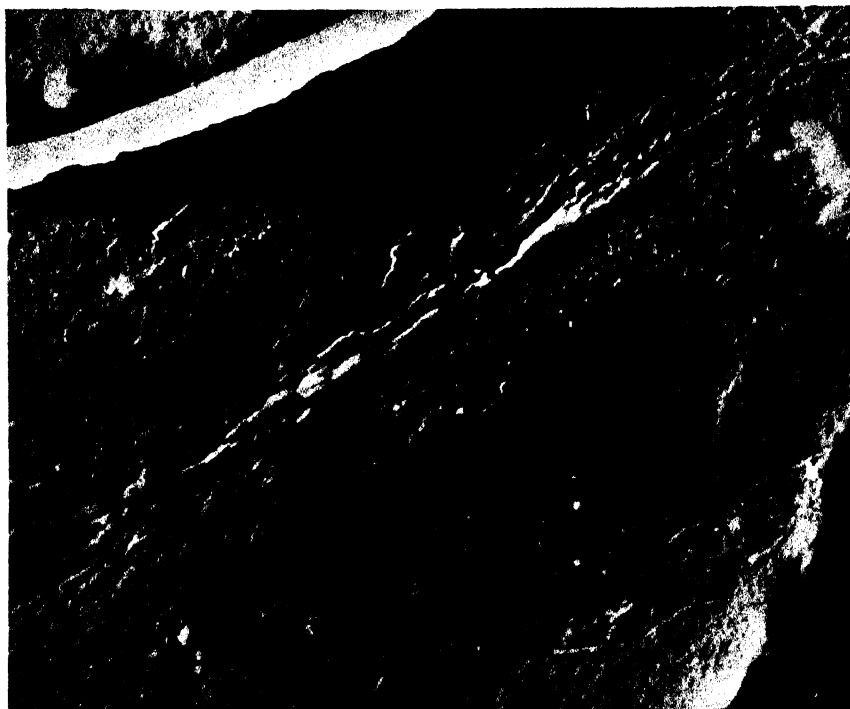


Fig. 8. An electron micrograph of sisal. The interior wall is heavily impregnated with lignin. Magnification = 20000  $\times$ .

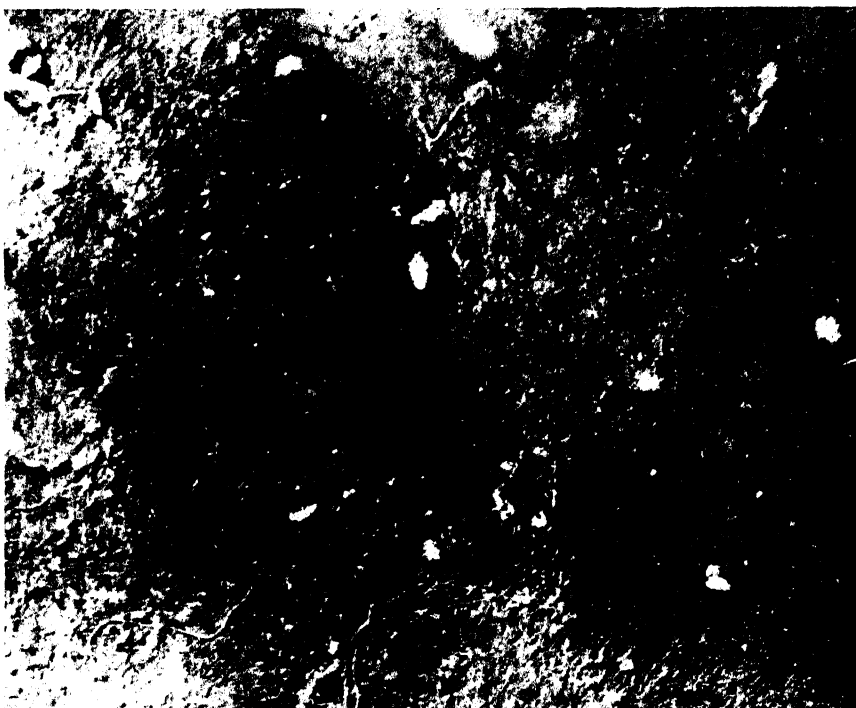


Fig. 9. An electron micrograph of sisal. A layer deposited deeper in the wall. Magnification = 20,000  $\times$ .

wall, on the other hand, are intertwined to form a loose network. Amorphous material incrusts the fibrils of both walls. Consisting principally of wax, pectin and hemicellulose, it can be dissolved by diluted  $\text{H}_2\text{O}_2$  or hypochlorite, without the cellulose structure being attacked.

### *Cotton*

There are more non-cellulose ingredients in the primary wall of cotton than of ramie (Fig. 4). As HESS and his collaborators pointed out, there is in fact so much wax, pectin, etc. in young cotton fibers that the X-ray diagram of cellulose is completely masked and does not become apparent until about thirty-six days after the petals have fallen. The cellulose diagram can, however, be shown as early as five days after falling of the petals, if the primary wall is first extracted with diluted alkali and then bleached. As will be seen from Fig. 4, these amorphous substances are embedded between the microfibrils, just as in the case of ramie.

Again as with ramie the fibrils in the secondary wall (Fig. 5) are arranged in parallel layers. Seen under the polarizing microscope, these layers do not follow a direction parallel to the fiber axis, but ascend spirally. Their direction of winding may be different, as for instance one layer will assume an S-shaped direction and the next one will be twisted in the opposite sense. This gives such fibers a very high degree of elasticity.)

### *Flax*

Flax fibers show more clearly than any others studied the fine structure of the fibrils composing them (Fig. 6). In the secondary wall (Fig. 7) these fibrils are obviously much more poorly arranged than in either ramie or cotton. They proceed, more or less intertwined, in the same general direction, but they do not form the closely packed parallel aggregates seen in these other fibers.

Flax provides an especially good material for testing the idea of FARR AND ECKERSON<sup>5</sup> that separate fibrils result from a linear joining of microscopic cellulose particles. These particles, which they thought they had discovered in the protoplasm of young cotton fibers, were supposed to be ellipsoidal ( $1.1 \mu$  thick and  $1.5 \mu$  long) and to be covered by a sheath of some non-crystalline substance. It was supposed that during cell growth the particles joined in chain-like fashion and later deposited as a whole in the cell wall. All the photographs of this paper conflict with this hypothesis. The fibrils do not have a thickness of  $1.1 \mu$ , nor is there any segmentation indicating that the fibrils consist of particles joined together. Flax fibrils too are of a uniform thickness of ca  $250 \text{ \AA}$ .

### *Sisal*

Lignin-containing fibers such as sisal, jute and wood present a picture different from that of the foregoing fibers.

Comparatively little is known as yet of the origin of lignin, of its chemical structure or the nature of its distribution in the cell wall. It is still undecided whether it occurs in a pure form or is combined chemically with other substances, but chemists, in increasing numbers, are assuming that most lignin is combined with carbohydrates. It is a fact that these substances are so intergrown with one another that on dissolving one the other still remains as a continuous cell-wall system.

Electron micrographs of sisal show that, as was the case with fiber types mentioned

above, non-cellulose substances are deposited between the micro fibrils. The interior walls are heavily impregnated with lignin (Fig. 8), the rough surface of which represents a very ragged picture. Remains of decayed protoplasm are often seen in these interior walls; in Fig. 8 they are apparent in the weakly developed fold that extends diagonally through the wall.

Layers deposited deeper in the wall (Fig. 9) are also completely filled with lignin, but their surface is smoother. As this figure indicates, the electron microscope shows hitherto unseen groups of fine pores penetrating through the cell wall. These pores undoubtedly provide the sole connection between cells after strong lignification has impeded early metabolism through their walls.

( A completely intact fibrillar structure of the cellulose is apparent after extracting lignin from the walls (Fig. 10). There are no indications that cellulose had been attacked during this extraction, and the thickness of the micro fibrils is not altered. It would thus appear that the fibrils are constructed of cellulose only and that sheaths of non-cellulose substances, as postulated by FARR AND WERGIN, are absent.

A skeleton of lignin is left behind after saccharification of the cellulose with concentrated acid (Fig. 11). This lignin evidently is in sheets and must have been deposited in this way rather than in the continuous fashion heretofore postulated. Such a manner of deposition parallels the layer-like deposition of cellulose. It would appear that after the cellulose structure has formed, each new wall layer is filled in with lignin. The electron micrographs demonstrate that the cellulose and lignin form two continuous structures, but that these two structures most intimately interpenetrate. This is the same structural principle as is presented by a wall of reinforced concrete. The skeleton, corresponding to the iron bars, is furnished by cellulose fibrils while lignin corresponds to the cement. This is well demonstrated in Fig. 11 where after dissolving cellulose from the wall, the former points of penetration of its fibrils leave fine pores through the lignin. This clever combination of high tensile strength cellulose and pressure resisting material (lignin) gives a wall offering the greatest mechanical stability.

#### *Wood fibers*

Wood, like sisal, has a high content of substances other than cellulose, as the following typical dry-weight analysis indicates:

Cellulose . . . . .	40-50%
Lignin . . . . .	20-30%
Hemicellulose . . . .	10-30%

As would be expected from such an analysis, electron micrographs show the fibrils in thick sections to be entirely covered by these non-cellulosic substances. Cellulose can, however, be seen in thinner sections (Fig. 12) and there too it consists of fibrils having a thickness of ca 250 Å. Lignin, shown in these sections, has an amorphous-granular structure that exhibits no intimate connection with cellulose since the fibrils appear unchanged by extraction (Fig. 13).

#### DISCUSSION

The electron micrographs of this paper indicate that in spite of their different origins (seed hairs, bast fibers, wood), all the plant cell walls examined have been

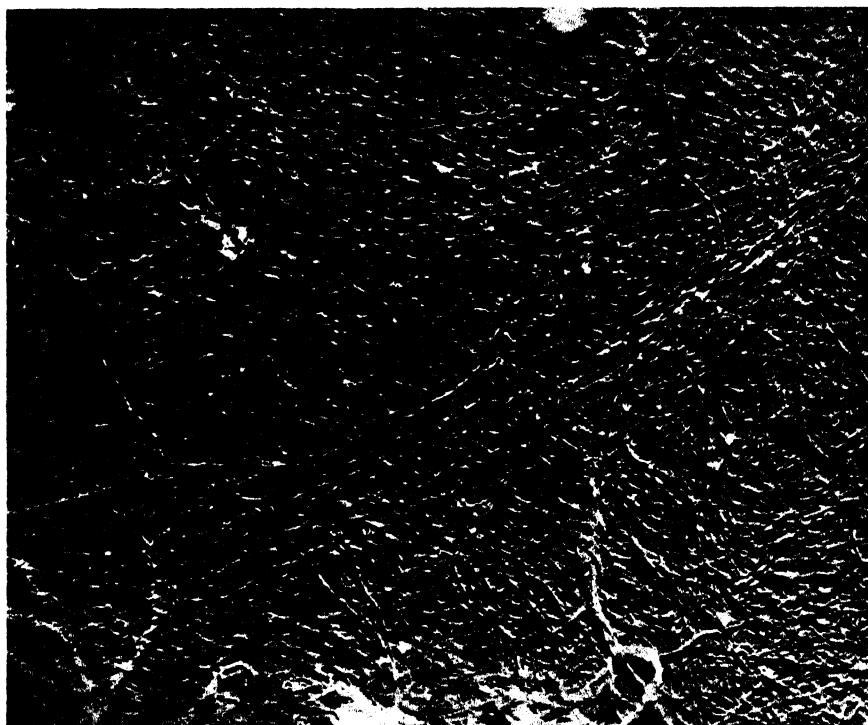


Fig. 10. An electron micrograph of sisal after extracting lignin from the wall. Magnification = 20 000  $\times$ .

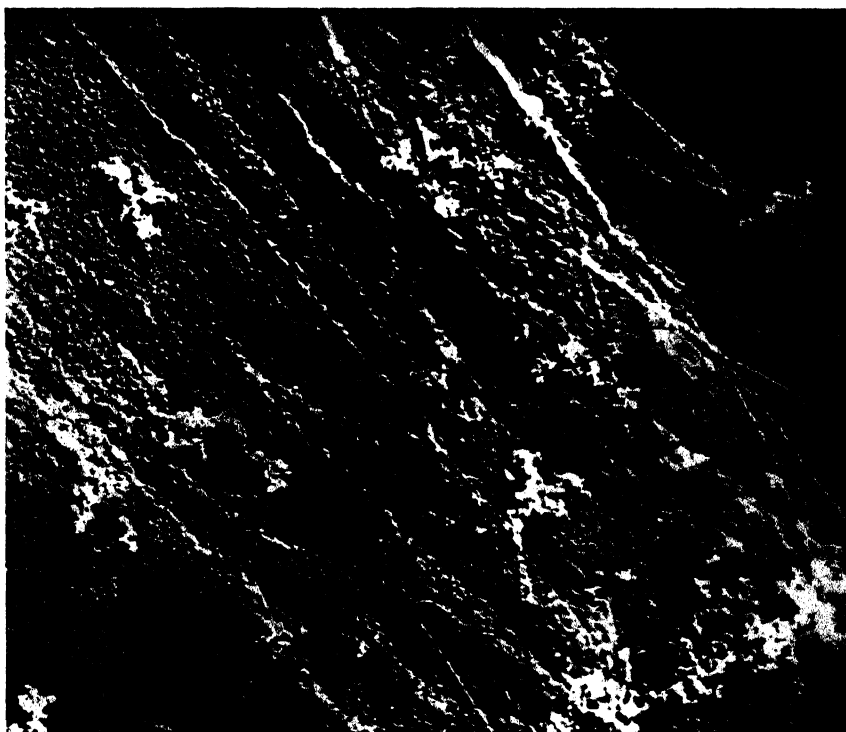


Fig. 11. An electron micrograph of a skeleton of lignin after saccharification of the cellulose with



Fig. 12. An electron micrograph of a wood fiber. Magnification = 20000  $\times$ .

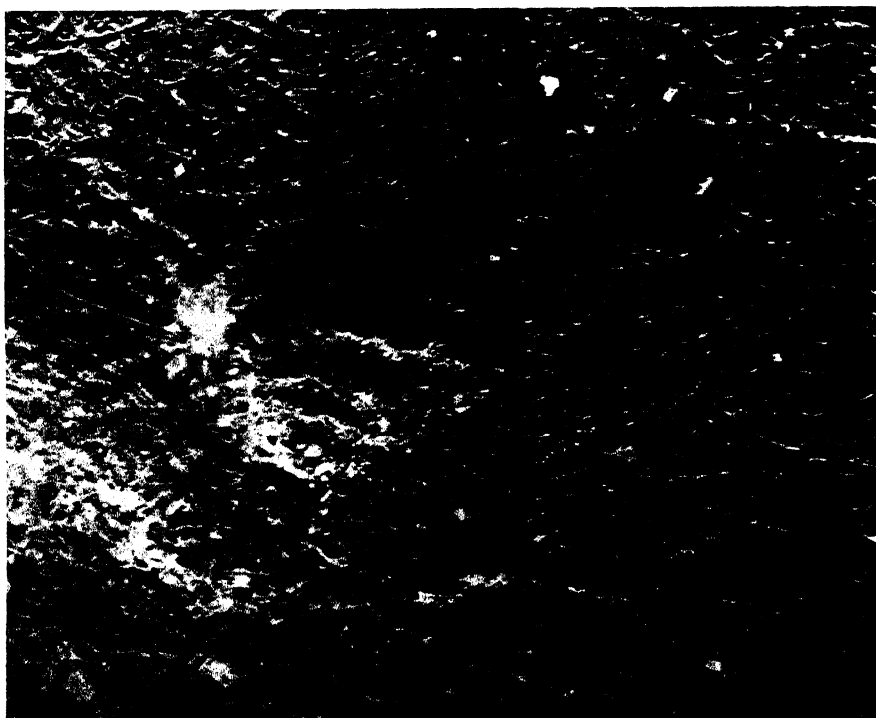


Fig. 13. An electron micrograph of a wood fiber after extracting lignin from the wall. Magnification = 20000  $\times$ .

constructed in the same general manner. Investigation of steps by which such cell walls have formed has now been carried out using the fast growing cells in coleoptiles, and these results will be published in a later paper.

This work was carried out while the writer was a Special Fellow of the American-Swiss Foundation for Scientific Exchange to which organization he wishes to express his deep gratitude. It was done in the laboratory of Dr R. W. P. WYCKOFF to whom the writer wishes to thank for many helpful discussions.

#### SUMMARY

Cell walls of ramie, cotton, flax, sisal and wood have been examined with the electron microscope. The Waring Blendor splits the fibers successfully and yields sections exhibiting an unaltered natural texture. In all these fibers, the cellulose consists of completely individualized micro-fibrils having a thickness of from ca 250–400 Å. In primary walls the micro-fibrils are intertwined to form a network, while in secondary walls they have a common direction and thus are arranged in a more or less parallel manner. Non-cellulose substances, such as lignin, pectin, wax and hemicellulose, are embedded between such fibrils and can be extracted from the cell walls to leave the fibrils undisturbed. Cellulose and non-cellulose each form an independent system.

#### RÉSUMÉ

Les parois des cellules de ramie, coton, lin, sisal et bois ont été soumises à un examen électronique. En employant une nouvelle méthode de coupage à l'aide du Waring Blendor, nous avons réussi à obtenir des coupures présentant une texture tout à fait naturelle. Il a été constaté que la cellulose dans toutes les fibres est formée de microfibrilles complètement individualisées et de 250–400 Å d'épaisseur. Dans la paroi primaire, ces microfibrilles sont entrelacées comme un filet, tandis que dans la paroi secondaire, elles sont toutes placées dans la même direction et arrangées plus ou moins parallèlement. Entre ces fibrilles se trouvent les substances étrangères à la cellulose, telles que lignine, pectine, cire et hémicellulose. Si on extrait ces substances de la paroi cellulaire, aucun changement des fibrilles n'apparaît, ce qui veut dire que les deux substances forment deux systèmes séparés, indépendants l'un de l'autre.

#### ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden Zellwände von Ramie, Baumwolle, Flachs, Sisal und Holz elektronenoptisch untersucht. Durch Anwendung einer neuartigen Schneide-Methode mit Hilfe des Waring Blendors, gelang es Schnitte mit völlig natürlicher Textur zu erhalten. Es zeigte sich, dass die Cellulose in allen Fasern in Form von 250–400 Å dicken, vollständig individualisierten Fibrillen ausgebildet ist. In der Primärwand sind diese Mikrofibrillen netzartig durcheinander verflochten, während sie in der Sekundärwand alle in der gleichen Richtung, mehr oder weniger parallel geordnet, verlaufen. Zwischen diesen Fibrillen sind die cellulosefremden Stoffe, wie z.B. Lignin, Pektin, Wachs und Hemicellulose eingelagert. Herauslösen derselben aus der Zellwand zeigt keine Veränderung der Fibrillen, was darauf hindeutet, dass beide Substanzen ein voneinander unabhängiges System bilden.

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## SUR L'INACTIVATION DE L'URÉASE PAR L'ISOCYANATE DE PHÉNYLE

par

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Dans un récent travail, HELLERMAN<sup>1</sup> a montré qu'une subunité d'uréase (21 300 g) conserve intégralement son activité en présence d'une molécule de p-chloromercuribenzoate; une deuxième molécule, par contre, provoque une inactivation presque totale. Mais, si l'uréase a été préalablement traitée par la porphyrindine diluée<sup>2</sup>, l'inactivation se produit dès que l'on a ajouté la première molécule du dérivé mercurique. Dans les deux cas, la cystéine permet de revenir à l'enzyme actif.

L'auteur conclut de ces observations que, parmi les 4 à 5 groupes thiol possédés par la subunité d'uréase, il faut distinguer:

1. Un groupe (a) très "réactif" dont la présence n'est pas indispensable à l'activité enzymatique-c'est lui qui donne la coloration caractéristique avec le nitroprussiate<sup>3</sup>-est oxydé par la porphyrindine diluée et se combine à la première molécule de p-chloromercuribenzoate.
2. Un groupe (b) de "réactivité" moindre dont le blocage provoque l'inactivation.
3. 2 à 3 autres groupes encore moins "réactifs" dont l'étude précise n'est pas poursuivie.

Le fait que l'activité de l'uréase n'est pas sous la dépendance de son groupe thiol le plus "réactif" est d'ailleurs présumé depuis longtemps. L'enzyme, en effet, n'est pas inactivé par une oxydation douce (ferricyanure<sup>4</sup>, porphyrindine et iodobenzoate dilués<sup>5</sup>) ni par des agents d'alkylation peu puissants (iodoacétate<sup>6</sup>) tandis qu'il l'est bien par une oxydation plus poussée (porphyrindine et iodobenzoate concentrés<sup>5</sup>) ou par des agents d'alkylation relativement énergiques (iodoacétamide<sup>6</sup>). HELLERMAN, en particulier, avait déjà signalé<sup>5</sup> l'obtention d'une préparation uréasique qui, traitée par la porphyrindine diluée jusqu'à disparition du test au nitroprussiate, était encore pleinement active.

Le principal intérêt de ce travail est donc qu'il attribue un rôle déterminant, non à l'ensemble des groupes thiol "non-réactifs" de l'uréase, mais à l'un d'entre eux seulement (groupe (b)). Cette conclusion est basée essentiellement sur le fait que l'inactivation, à partir de l'instant où elle commence, devient totale par addition d'une molécule exactement de p-chloromercuribenzoate.

Remarquons toutefois qu'une telle conclusion n'est valable que si le p-chloromercuribenzoate se combine stoechiométriquement aux -SH (b). Or, les observations d'ANSON<sup>7</sup>, quoique de nature assez indirecte, suggèrent que le dérivé mercurique s'unit mal aux -SH "non-réactifs" des protéines. Il n'est donc pas certain qu'au moment où l'activité uréolytique devient nulle, les -SH (b) soient complètement bloqués. Si ce

doute s'avérerait justifié, d'ailleurs, la notion même de -SH (b) perdrait beaucoup de son intérêt et les expériences d'HELLERMAN montreraient simplement que le p-chloromercuribenzoate inactive l'enzyme quand il commence à atteindre ses -SH "non-réactifs".

Au lieu de se fier simplement aux quantités d'inhibiteur mises en œuvre, il apparaît ainsi beaucoup plus sûr de doser directement les groupes thiol de la protéine fermentaire aux divers stades de son inactivation. C'est d'ailleurs ce qu'a fait BAILEY<sup>8</sup> dans une étude similaire sur la myosine.

Nous avons donc repris le travail d'HELLERMAN et, au moment de choisir l'inhibiteur, nous nous sommes laissés guider par les considérations suivantes:

1. Il est désirable que la substance inhibitrice, tout en étant bien spécifique des -SH, possède une affinité nettement plus grande pour les -SH "réactifs" que pour les "non-réactifs". Les blocages des deux formes sont alors largement échelonnés dans le temps et leurs conséquences respectives sont aisément observables.

2. La présence de cette substance ne doit pas interdire la détermination quantitative des groupes restés libres.

Le cétène et l'isocyanate de phényle, dont nous avons récemment étudié l'action sur les groupes thiol protéiques<sup>9</sup>, semblent satisfaire à ces deux conditions. Le cétène, toutefois, s'est révélé d'un emploi difficile, car il risque d'entraîner des inactivations non-spécifiques par l'acidité qu'il développe et la nécessité où il met de dialyser les préparations. Nous verrons tout-à-l'heure, de plus, qu'il s'unit moins bien aux -SH "réactifs" que l'isocyanate. C'est donc cette dernière substance seulement qu'en fin de compte nous avons utilisée.

## TECHNIQUES UTILISÉES

### I. DÉTERMINATION DE L'ACTIVITÉ URÉOLYTIQUE

La conduite du test, la détermination colorimétrique de l'ammoniaque libérée et le dosage de l'azote protéique dans les solutions uréasiques ont été réalisés selon SUMNER<sup>10</sup>. Notons simplement que les préparations très impures d'uréase donnent naissance à un trouble au moment où l'on ajoute le réactif de NESSLER. Le milieu a donc été systématiquement déféqué à l'acide trichloracétique avant dosage<sup>11</sup>. D'autre part, une courbe de référence (photocolorimètre, cuve de 1 cm, filtre Wratten No 47) montre que, dans nos conditions expérimentales, la loi de BEER est parfaitement satisfaite entre 350 et 650  $\mu\text{g}$  de  $\text{N-NH}_3$ .

Nous appelons, comme de coutume, unité d'uréase ( $U.$ ) la quantité d'enzyme qui, à 20°, à  $\text{pH} = 7.0$  et en présence d'un fort excès d'urée, met en liberté 1 mg de  $\text{N-NH}_3$  pendant les 5 premières minutes. Ayant mesuré, d'autre part, la teneur des préparations en azote protéique ( $N.P.$ ), nous pouvons déterminer leur pureté ( $U.g.$ ) qui, par convention, est le nombre d'unités par g de protéine (ou 0.17 g de  $N.P.$ )..

### II. PRÉPARATION DE L'URÉASE

Deux techniques, d'ailleurs analogues, ont été indiquées jusqu'ici pour la préparation de l'uréase pure: D'une part, la technique initiale de SUMNER<sup>10</sup> qui prévoit la cristallisation directe de l'enzyme à partir d'un extrait hydroacétonique de farine de *Canavalia ensiformis*, suivie d'une recrystallisation éventuelle selon DOUNCE<sup>12</sup>. D'autre part, celle préconisée par HELLERMAN<sup>1</sup> qui consiste à précipiter tout d'abord, à l'état très impur, la quasi-totalité de l'uréase contenue dans l'extrait précédent, puis à purifier l'enzyme par dissolutions fractionnées et cristallisation.

Après de nombreuses cristallisations, la pureté de l'uréase semble pouvoir être portée à 130000  $U.g.$  et c'est ce chiffre qui servira de base à nos calculs ultérieurs. HELLERMAN signale être arrivé à une pureté de 105000  $U.g.$ , mais l'ensemble de son travail paraît avoir été effectué sur des préparations dont la pureté était de 75000  $U.g.$  et au-dessous.

*Bibliographie p. 33.*

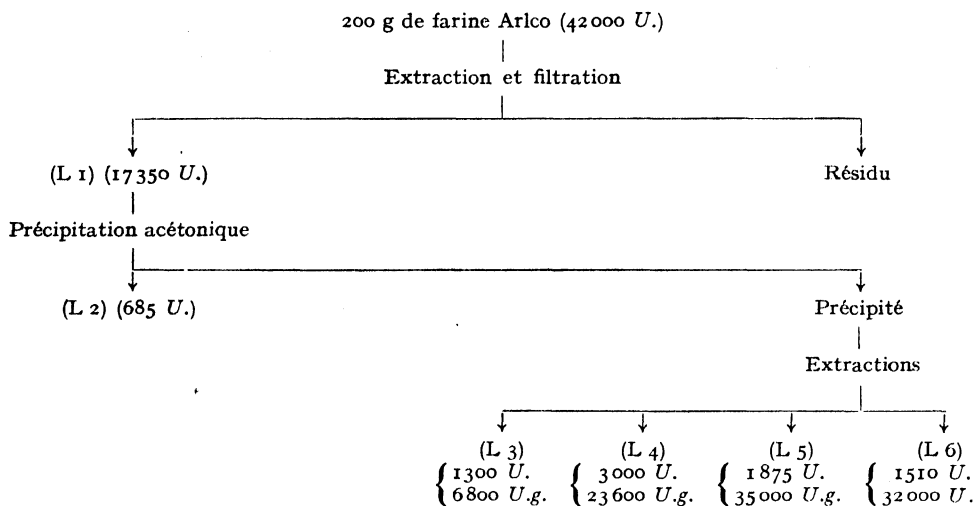


La méthode de cristallisation directe ne donnant pas de bons résultats avec notre échantillon de *Canavalia*\*, nous avons suivi la technique d'HELLERMAN qui semble d'ailleurs être mieux adaptée à la préparation de quantités importantes d'enzyme. Voici notre mode opératoire (Schéma I).

200 g de farine Arlco sont triturés au mortier avec 1 l d'acétone à 31.5 %\*\* (température 22° C). La suspension est essorée sur filtre à plis, puis le résidu est séché aussi bien que possible sur Büchner. Les liquides sont réunis et additionnés d'acétone pure jusqu'à ce que la concentration du solvant (vol/vol) atteigne 42 %. Après une nuit passée à la glacière, le mélange est centrifugé rapidement. Le liquide surnageant (L 2) ne contient presque plus d'uréase. Le précipité est lavé par 1.6 ml d'eau glacée que l'on sépare immédiatement par centrifugation. On obtient ainsi un liquide (L 3) actif, mais très impur. Il n'est donc pas utilisé. Le culot de centrifugation est ensuite traité par 2.5 ml d'eau et ce mélange est laissé 5 h à la glacière où il est fréquemment agité. Au bout de ce temps, il est centrifugé au froid jusqu'à obtention d'un liquide (L 4) clair (4 h environ). Le résidu solide est encore extrait 2 fois à l'eau dans les mêmes conditions (liquides (L 5) et (L 6)). Ce sont ces derniers liquides que nous avons acylés.

#### SCHEMA I

##### PRÉPARATION DES SOLUTIONS D'URÉASE



Les chiffres du schéma I font apparaître que les liquides (L 4), (L 5) et (L 6) contiennent, outre de l'uréase, des quantités encore notables de protéines étrangères. Toutefois, comme nous le verrons par la suite, ces protéines ne sont pas gênantes car elles ne possèdent aucun groupe thiol "réactif" ou "non-réactif". Les liquides précédents ont donc pu être utilisés directement sans purification supplémentaire.

#### III. DOSAGE DES GROUPES THIOL PROTÉIQUES

Dans la plupart des cas, nous avons dosé les -SH totaux de la protéine fermentaire. L'oxydation au ferricyanure en présence de 1.75 % de dodécylsulfate a alors été utilisée

\* Nous sommes heureux de remercier ici très vivement le Prof. SUMNER de l'envoi généreux d'un échantillon de farine Arlco avec lequel une grande partie de ce travail a été effectuée.

\*\* L'acétone est préalablement distillée sur chlorure de Ca, puis sur chaux.

(Pour plus de détails expérimentaux, voir<sup>9</sup>). Quant aux -SH "réactifs" de la protéine native, ils ont été déterminés également par le ferricyanure, mais en l'absence de détergent. Avant de procéder au dosage colorimétrique, nous avons alors déféqué le milieu par l'acide tungstique<sup>9</sup>.

## RÉSULTATS EXPÉRIMENTAUX ET DISCUSSION

### I. CARACTÉRISTIQUES DES PRÉPARATIONS URÉASIQUES UTILISÉES

Les préparations d'uréase obtenues par la technique qui vient d'être décrite possèdent, nous l'avons vu, une pureté comprise entre 32 000 et 35 000 *U.g.* Leur teneur réelle en enzyme est calculée en admettant que 1 g d'uréase pure développe une activité de 130 000 *U.* Les -SH "réactifs" et totaux ont été trouvés correspondre, respectivement, à 0.58 et 2.75 g de cystéine pour 100 g d'enzyme.

Le chiffre de 0.58% semble en bon accord avec celui des autres auteurs, puisqu'il indique la présence de 1.02 groupe thiol dans 21 300 g d'enzyme. Il est cependant trop fort, car, par suite sans doute d'une mauvaise précipitation tungstique de l'uréase ou de l'une des protéines qui l'accompagnent, la liqueur soumise au dosage colorimétrique présente un trouble assez notable. Nous pensons donc que nos préparations enzymatiques ont subi une légère autoxydation qui, d'ailleurs, n'influe pas sur leur activité. Si cette hypothèse est exacte, il conviendrait alors de relever quelque peu le chiffre de 2.75% trouvé pour les -SH totaux. Une teneur initiale de 2.85%, correspondant à 5 -SH par subunité d'uréase, a donc été adoptée.

### II. ACYLATIONS PAR DES QUANTITÉS CONNUES D'ISOCYANATE ET DE CÉTÈNE

Jusqu'ici, les protéines ont été généralement traitées par un très gros excès de cétène ou d'isocyanate de phényle et les progrès de l'acylation ont été suivis en fonction du temps. Cette manière de faire était d'ailleurs justifiée, puisque les groupes protéiques étudiés (-NH<sub>2</sub>, -OH phénoliques) se combinent assez lentement à l'agent acylant pour que celui-ci soit en majeure partie hydrolysé. L'acylation des -SH "réactifs" se présente cependant sous un jour différent, puisqu'elle est presque instantanée. Il n'est donc pas interdit de chercher à la placer sur le plan stoechiométrique, c'est-à-dire à connaître le nombre de molécules de l'agent qu'il faut mettre en œuvre pour bloquer 1 -SH "réactif". Une telle étude nous a paru devoir faciliter considérablement l'approche expérimentale de notre principal objectif. Elle a été effectuée sur les -SH "réactifs" de l'albumine d'œuf dénaturée.

Un poids connu d'isocyanate est dissous dans l'acétone pure et parfaitement anhydre, puis cette solution est ajoutée, lentement et en agitant bien, à une dispersion d'albumine dans le dodécylsulfate à 1.75%. Quant au cétène, on le fait barboter dans l'éther anhydre et privé de peroxydes par le sulfate ferreux. La teneur en cétène est déterminée sur une partie aliquote que l'on fait réagir soit avec un excès de soude M/100, soit avec une solution éthérée d'aniline. Dans le premier cas, on titre en retour par SO<sub>4</sub>H<sub>2</sub> M/100 en présence de phtaléine du phénol. Dans le second, on lave l'acétanilide par l'acide acétique dilué, on le sèche et on le pèse\*. La solution de cétène qui, rappelons-le, doit-être

\* On tient compte de la solubilité de l'acétanilide en partant d'un mélange connu de cette substance et d'aniline que l'on traite par les mêmes quantités d'éther et d'acide acétique que précédemment.

absolument dépourvue de peroxydes, est alors ajoutée à la dispersion protéique comme il est indiqué plus haut. Le blocage des -SH est suivi par la technique habituelle<sup>9</sup>. Un essai-témoin nous a montré qu'il n'était pas nécessaire de conduire l'acylation sous azote car les -SH, même "réactifs", ne s'autoxydent pas sensiblement pendant le traitement. Les résultats expérimentaux sont donnés dans le Tableau I.

TABLEAU I  
ACYLATION, PAR DES QUANTITÉS CONNUES DE CÉTÈNE OU D'ISOCYANATE, DES -SH "RÉACTIFS"  
DE L'ALBUMINE DÉNATURÉE

Albumine mise en jeu (mg)	Milli-équiv. de -SH "réactifs"	Agent acylant		Rapport (4)/(2)	Acylation (%)
		Nature	Millimol utilisées		
(1)	(2)	(3)	(4)	(5)	(6)
140	0.0116	Isocyanate	0.0116	1.00	76
140	0.0116	"	0.0134	1.16	100
140	0.0116	"	0.0232	2.00	100
71	0.0058	Cétène	0.0118	2.04	19
56	0.0046	"	0.0207	4.5	31
140	0.0116	"	0.132	11.4	52
89	0.0074	"	0.230	31.0	64

L'examen des chiffres du Tableau I montre clairement que les -SH "réactifs" de l'albumine sont totalement bloqués par l'addition de 1.16 fois la quantité théorique d'isocyanate. Par contre, plus de 30 fois la quantité théorique de cétène ne provoque que 64% d'acétylation seulement. Cette différence provient-elle d'une affinité plus grande du cétène pour les molécules d'eau ou trouve-t-elle son origine dans une affinité plus grande de l'isocyanate pour les -SH protéiques? La question mériterait d'être discutée. Nous nous contenterons simplement d'indiquer ici que le comportement très favorable de l'isocyanate nous a incités à utiliser exclusivement cet agent d'acylation dans nos expériences sur l'uréase.

### III. ACTION DE L'ISOCYANATE DE PHÉNYLE SUR L'URÉASE

Voici tout d'abord le protocole expérimental de l'un de nos essais les plus caractéristiques:

A 2.85 ml d'une liqueur contenant 15.8 mg d'uréase (pureté: 35 000 *U.g.*), on ajoute successivement des quantités connues d'isocyanate en solution acétonique. Aucune précipitation de diphenylurée ne se produit et le milieu reste parfaitement limpide. Des échantillons sont prélevés avant chaque nouvelle addition, sur lesquels on dose l'activité uréolytique et les groupes thiol totaux. L'acétone seule et l'agitation ne provoquent aucune inactivation.

Les résultats de cette étude sont donnés sous forme graphique par les Figs 1 et 2.

Ces figures suggèrent les quelques remarques suivantes:

1. Dès que l'on traite la solution d'uréase par l'isocyanate, certains -SH disparaissent rapidement (Fig. 1). C'est ainsi que 2.25 équivalents de l'agent acylant bloquent 1 -SH sur 5. Nous assistons, de toute évidence, à l'acylation des -SH (a) selon HELLERMAN qui, étant "réactifs", se combinent presque stoechiométriquement à l'isocyanate.

Le blocage ainsi constaté n'exerce aucune influence notable sur l'activité enzymatique. Nous confirmons donc de façon tout-à-fait nette les résultats obtenus par HELLERMAN dans ce domaine et les suggestions de ses prédécesseurs.

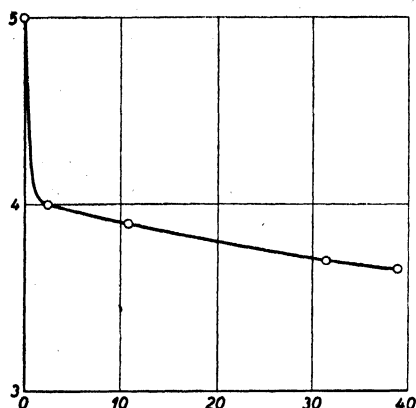


Fig. 1. Variation du nombre de groupes thiol de l'uréase pendant l'acylation.

En abscisses: Nombre d'équivalents isocyanate ajoutés (calculés par rapport aux-SH (a) de l'uréase).

En ordonnées: Nombre de groupes -SH totaux dans 21 300 g d'uréase.

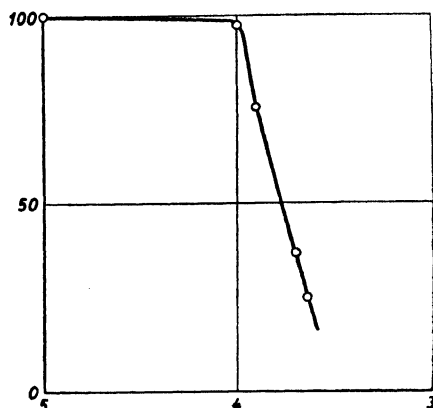


Fig. 2. Activité de l'uréase en fonction de sa teneur en -SH totaux.

En abscisses: Nombre de groupes -SH totaux dans 21 300 g d'uréase.

En ordonnées: Activité % (l'activité initiale est prise comme base 100).

2. Si l'on poursuit l'addition d'isocyanate, la teneur en -SH totaux baisse beaucoup moins vite (Fig. 1). Il faut en effet à ce moment 29 équivalents d'isocyanate pour faire disparaître 0.3 -SH, donc près de 50 fois plus qu'auparavant. Les groupes -SH qui sont alors bloqués sont ainsi nettement moins "réactifs" que les précédents.

3. Au cours de cette deuxième phase, le ferment s'inactive brusquement (Fig. 2). Il ne paraît pas probable que l'excès d'isocyanate agisse sur d'autres groupes que les -SH, car, en milieu non-tamponné et légèrement acide, les groupes  $-NH_2$  et  $-OH$  phénoliques s'acylent très lentement, même quand ils se trouvent en présence de quantités considérables d'agent acylant<sup>9</sup>. Dans notre cas, ces phénomènes parasites sont donc certainement négligeables et l'action de l'isocyanate sur les groupes -SH "non-réactifs" semble bien être la cause déterminante de l'inactivation.

4. La courbe de la Fig. 2 met nettement en évidence cette chute d'activité uréolytique dès que les -SH "non-réactifs" commencent à s'acyler. Cette courbe, il ne nous a malheureusement pas été possible de la tracer jusqu'à disparition totale de l'activité enzymatique. Sa pente, toutefois, est tellement accusée qu'elle suggère que l'inactivation totale est atteinte bien avant que le deuxième -SH soit complètement bloqué. Nos essais conduisent donc, sur ce point, à des résultats un peu différents de ceux d'HELLERMAN, car ils ne font pas apparaître de façon aussi nette la nécessité de distinguer, parmi les -SH "non-réactifs" de l'uréase, des groupes (b) particuliers et de leur conférer une importance physiologique spéciale. Ce désaccord s'explique d'ailleurs aisément si l'on admet (voir l'introduction) que le p-chloromercuribenzoate ne se combine pas stoechiométriquement aux -SH non-réactifs.

Quoi qu'il en soit d'ailleurs, un fait fondamental semble bien acquis: L'uréase perd son activité au moment où l'on commence à bloquer ses -SH "non-réactifs". Il faut

alors noter que cette "non-réactivité" provient soit de l'existence, au sein de l'uréase native, de liaisons structurales labiles dans lesquelles les groupes se trouvent impliqués, soit d'un empêchement stérique quelconque. Dans les deux cas, il paraît difficile de croire que ces groupes "non-réactifs" puissent jouer par eux-mêmes un rôle précis dans l'activité enzymatique, en facilitant, par exemple, la formation du complexe enzyme-substrat.

Mais, dans les deux cas également, la protéine native subit certainement des modifications importantes de structure avant que ses groupes "non-réactifs" soient bloqués par la substance chimique mise en œuvre. Peut-être, ces modifications qui se produisent soit au moment de la rupture des liaisons labiles éventuelles, soit au moment où la substance chimique fraye son chemin jusqu'aux groupes difficilement accessibles, sont-elles de nature à entraîner la perte de l'activité. Si cette hypothèse est exacte, le blocage des groupes aurait un caractère secondaire et le phénomène réellement important consisterait en une série de dénaturations partielles intervenant à leur niveau. Rien n'empêche, d'ailleurs, de croire que les dénaturations soient suffisamment localisées, au début tout-au-moins, pour être réversibles.

Nous pensons que cette hypothèse devrait être envisagée *toutes les fois que l'activité physiologique d'une protéine semble dépendre de l'intégrité de l'un de ses groupes "non-réactifs"*. Sans en entamer ici la discussion générale, qui est sans doute encore impossible aujourd'hui, notons que le cas de la myosine, autre "sulfhydryl enzyme", doit être provisoirement réservé tant que les contradictions existant entre les résultats de BAILEY<sup>8</sup> et ceux de SINGER<sup>13</sup> ne seront pas levées. Signalons aussi, qu'en dehors des groupes thiol, on attribue souvent à certains autres groupes protéiques (phénol, amine) un rôle précis dans l'activité physiologique des protéines. Il est alors curieux de constater que ces derniers groupes sont également susceptibles, grâce à leur hydrogène mobile, d'entrer dans des liaisons structurales labiles (phénol<sup>14</sup>, amine<sup>15</sup>). Les groupes phénol de la pepsine, par exemple, sont doués d'une très faible "réactivité"<sup>14</sup> et il n'est probablement pas sans intérêt de rapprocher ce fait des résultats expérimentaux classiques d'HERRIOTT<sup>16</sup> sur l'inactivation de l'enzyme par le cétène.

## RÉSUMÉ

L'acylation, en milieu légèrement acide, de 1 -SH "réactif" de l'albumine d'œuf dénaturée nécessite l'emploi de 1.16 mol d'isocyanate de phényle (en solution acétonique) et plus de 50 mol de cétène (en solution étherée). Un précédent travail a, d'autre part, montré que l'acylation des -SH "réactifs" des protéines est presque instantanée, tandis que celle des -SH "non-réactifs" est au contraire très lente.

Partant de ces observations, nous avons traité de l'uréase par des quantités connues d'isocyanate en solution acétonique et nous avons mesuré la perte progressive d'activité subie par l'enzyme ainsi que le blocage de ses groupes -SH. Nous confirmons que l'uréase est encore pleinement active quand ses -SH "réactifs" sont bloqués. L'inactivation débute brusquement dès que les -SH "non-réactifs" se trouvent atteints. Elle semble être totale avant que les -SH (b) d'HELLERMAN soient complètement acylés.

Pour les protéines qui, comme l'uréase, *s'inactivent quand on bloque certains de leurs groupes "non-réactifs"*, une hypothèse est présentée d'après laquelle l'inactivation résulterait, non du blocage lui-même, mais des changements structuraux réversibles survenant au niveau de ces groupes au moment où on les force à réagir.

## SUMMARY

The acylation, in weakly acid medium, of one "reactive" -SH of denatured egg albumin requires 1.1 mol phenyl isocyanate (in acetone solution) and more than 50 mol ketene (in ethereal solution).

*Bibliographie p. 33.*

A previous investigation has shown that the acylation of the "reactive" -SH of proteins is almost instantaneous, while that of "non-reactive" -SH is very slow.

Starting from these observations, we have treated urease with known quantities of isocyanate in acetone solution and have measured the progressive loss of activity at the same time as the blockage of its -SH groups. We confirm that urease is still fully active when its "reactive" -SH groups are blocked. Inactivation begins suddenly when the "non-reactive" -SH groups are attacked. It seems to be complete even before the -SH (b) groups of HELLERMAN are completely acylated.

For proteins such as urease, *which are inactivated when certain of their "non-reactive" groups are blocked*, a hypothesis is presented according to which inactivation results not from the blockage itself, but from reversible structural changes at the site of these groups when they are caused to react.

### ZUSAMMENFASSUNG

Die Azylierung von einer "aktiven" -SH-Gruppe des denaturierten Eialbumins in schwach saurem Milieu erfordert die Anwendung von 1.16 Mol Phenylisocyanat (in acetonischer Lösung) und mehr als 50 Mol Ketten (in ätherischer Lösung). In einer vorgehenden Arbeit wurde andererseits gezeigt, dass die Azylierung "aktiver" -SH-Gruppen beinahe momentan erfolgt, während die der "inaktiven" -SH-Gruppen im Gegensatz dazu sehr langsam verläuft.

Auf Grund dieser Beobachtungen behandelten wir Urease mit bekannten Isocyanatmengen in acetonischer Lösung und massen den steigenden Aktivitätsverlust, den das Enzym bei der Blockierung seiner -SH-Gruppen erleidet. Wir bestätigen, dass die Urease noch vollkommen aktiv ist, wenn ihre "aktiven" -SH-Gruppen blockiert sind. Die Inaktivierung beginnt danach plötzlich, sobald die "inaktiven" -SH-Gruppen angegriffen werden. Sie scheint bereits vollständig zu sein, bevor die -SH (b)-Gruppen von HELLERMAN vollständig acyliert sind.

Für die Eiweisskörper, die — wie die Urease — *inaktiviert werden, wenn man gewisse ihrer "inaktiven" Gruppen blockiert*, wird eine Hypothese aufgestellt, nach welcher die Inaktivierung nicht von der Blockierung selbst, sondern von reversiblen Strukturveränderungen im Niveau dieser Gruppen herrührt, die auftreten, sobald man sie zur Reaktion bringt.

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# THE PLASMA PROTEIN EQUILIBRIUM FACTOR: A NEW CHEMICAL DETERMINATION, OF CLINICAL SIGNIFICANCE

by

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In this communication there will be described a new factor which can be used where the estimation of the plasma proteins is made in the study of the systemic reaction to organic changes in the body. This factor has proved to be more reliable and more informative than the classical albumin/globulin ratio.

The new factor is related to the equilibrium constant of the reaction which depends on the hypothesis that in plasma, in addition to free albumin, globulin and fibrinogen, there is a "combined-protein" molecule of bound albumin-globulin-fibrinogen, the four molecules being assumed to be in equilibrium.

## INTRODUCTION

The existence of a combined-protein molecule in plasma has been postulated by many writers and denied by others.

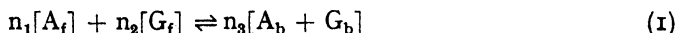
When we failed in our endeavours to obtain a formula by which to correlate a physical property (viscosity) with the plasma chemical composition and especially the protein fractions, we again considered the possibility of the existence of such a combined-protein molecule. During the analysis of the experimental results from this aspect a new factor was derived whose clinical significance was soon apparent.

The details of the analysis have been given in a previous publication (HARKNESS AND WHITTINGTON)<sup>1</sup>; only the essentials are repeated here.

(The plasma proteins were fractionated by the classical sodium sulphate method of HOWE<sup>2</sup>, the nitrogen being estimated by a micro-Kjeldahl technique. The fact that these fractions were not pure albumin and globulin was fully realized, but this was the best method available to us. For simplicity, at this stage, the subfractions are ignored and the fractions regarded as homogeneous.)

In support of the hypothesis of the existence of a combined-protein molecule, there is evidence that the fractions behave as would be required by the law of mass action.

If, in serum, as in equation (1),  $n_1$  molecules of free albumin and  $n_2$  molecules of free globulin are in equilibrium with  $n_3$  molecules of bound albumin plus bound globulin,



then the law of mass action would require that the second equation be satisfied

$$\left[ \frac{A_f}{M_A} \right]^{n_1} \times \left[ \frac{G_f}{M_G} \right]^{n_2} = k \left[ \frac{A_b + G_b}{M_P} \right]^{n_3} \quad (2)$$

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where  $k$  is the true "equilibrium constant" of the reaction and  $M_A$ ,  $M_G$ , and  $M_P$  are the molecular weights of the albumin, globulin and combined-molecule respectively.

By the introduction of further assumptions, this can be reduced to equation (3)

$$A^a \cdot G^{1-a} = K (A + G) \quad (3)$$

where  $A$  and  $G$  are albumin and globulin as measured in g per 100 ml as fractionated.

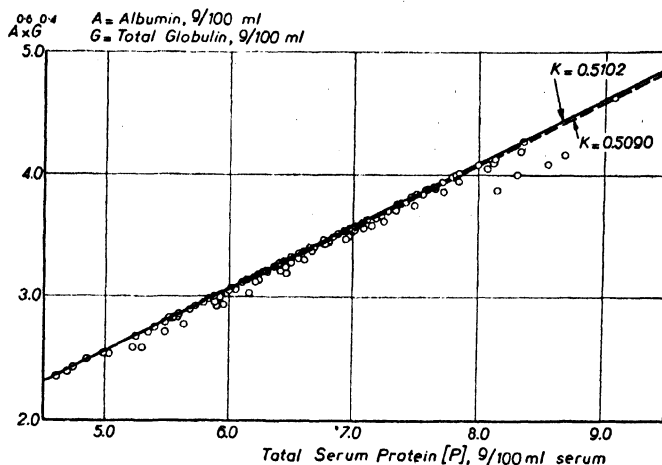


Fig. 1. The serum-protein line ( $\alpha = 0.6$  empirical)

If the hypothesis is correct, then there should be a value for ' $\alpha$ ' which would allow  $K$  to be approximately constant, and such a value has been found where  $\alpha = 0.59$

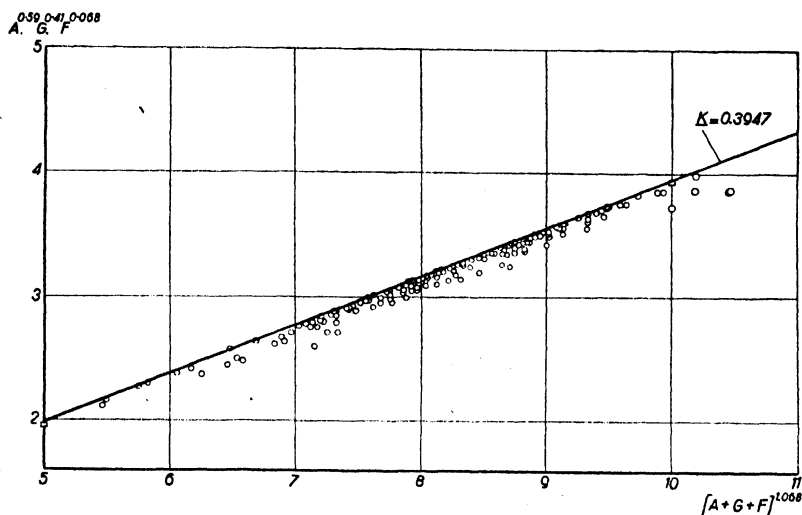


Fig. 2. The plasma-protein line

(empirically  $\alpha = 0.60$ , but calculation showed that theoretically  $\alpha$  should equal 0.59).

Fig. 1 shows  $A^a \cdot G^{1-a}$  plotted against  $(A + G)$  for 212 tests, and the line  $K$  is obviously nearly a straight line, as required by the hypothesis.

References p. 43.



The hypothesis was then extended to plasma, with the equilibrium equation becoming as in (4)

$$A^a \cdot G^{1-a} \cdot F^\beta = K (A + G + F)^{1+\beta} \quad (4)$$

The diagram for plasma corresponding to Fig. 1 for serum, is shown in Fig. 2, and here it is seen that  $K$  is more complicated. While there appears to be an upper limiting value for  $K$  at 0.3947, there are numerous and considerable departures from this line.

Consideration of our clinical material soon showed that there was a relation between the clinical condition and the departure of the  $K$  value from the straight line.

The clinical material consisted of cases in which the plasma proteins were changing as part of the systemic reaction to organic changes in the body, *e.g.* tuberculosis, sepsis, rheumatism, malaria, malignant growths, etc. Those conditions in which the proteins vary as a result of primary liver or kidney lesions are omitted as insufficient numbers of such cases have been studied to warrant an opinion.

#### ESTIMATION OF $K$

When the values of  $A$ ,  $G$ , and  $F$  are known (in g/100 ml)  $K$  can be calculated from equation (5)

$$K = \frac{R^{a+\beta} \cdot S}{[R + S(1 + R)]^{1+\beta}} \quad (5)$$

where

$$R = A/G, S = A/F, a = 0.59 \text{ and } \beta = 0.068.$$

To eliminate this calculation, the diagram of Fig. 3 was constructed whereby  $K$  can be read off by interpolation when  $R$  and  $S$  are known.

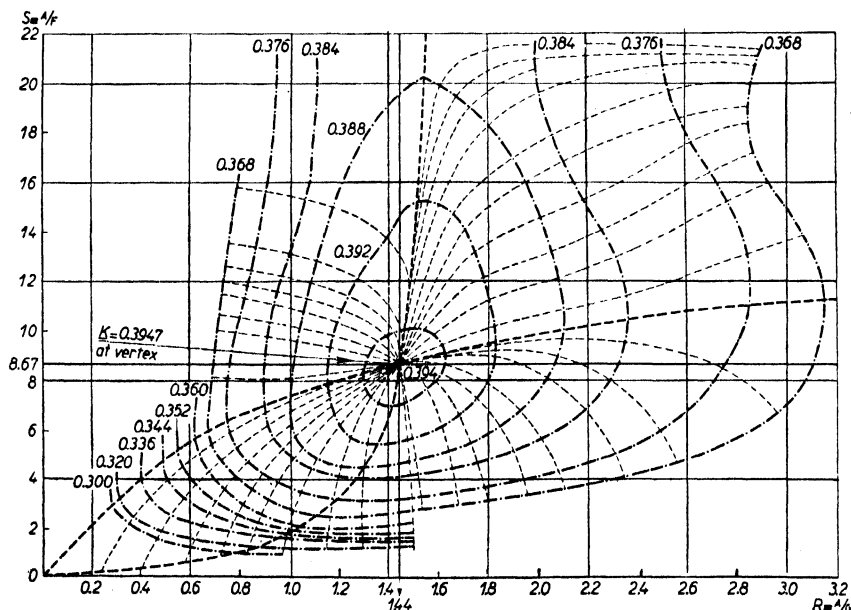


Fig. 3. Nomogram for estimation of  $K$  from  $R \equiv A/G$  and  $S \equiv A/F$ . Contours of equal  $K$  values. Orthogonals of maximum change in  $K$  with least change in protein fractions.

References p. 43.

Fig. 3 has also been of great assistance in the understanding of the changes in  $K$  and in the differentiation between  $K$  values of equal numerical value but different clinical significance.

(Fig. 3 can best be appreciated by regarding it as similar to a contour map, with contours here of equal  $K$  values and the peak at 0.3947. Drawn at right-angles to the contours are the orthogonals.)

#### RELATION OF $K$ TO THE CLINICAL CONDITION

In health,  $R$  ( $= A/G$ ) and  $S$  ( $= A/F$ ) are both high and  $K$  values lie in the upper right portion of Fig. 3 and the  $K$  values are numerically low.

Generally, it appears that as a response to the onset of infection and organic bodily changes the fibrinogen and/or globulin increases while the albumin later decreases. These changes cause  $K$  to move to the left and downwards, and  $K$  first *increases* numerically and then *decreases*.

With clinical improvement,  $K$  retraces its path towards the right, increasing numerically and then decreasing.

From our experience in about 285 cases, we believe that the introduction of the  $A/F$  ratio has produced in the  $K$  factor a value which is superior to the  $A/G$  ratio as an indication of the reaction of the body to associated organic changes.

a. Outside the upper right section of normality, cases of equal clinical severity were associated with approximately the same numerical value of  $K$  although the situation (and  $A/G$  ratio) in Fig. 3 could be highly variable.

This point is well exemplified by Fig. 4 in which the average  $K$  values are plotted

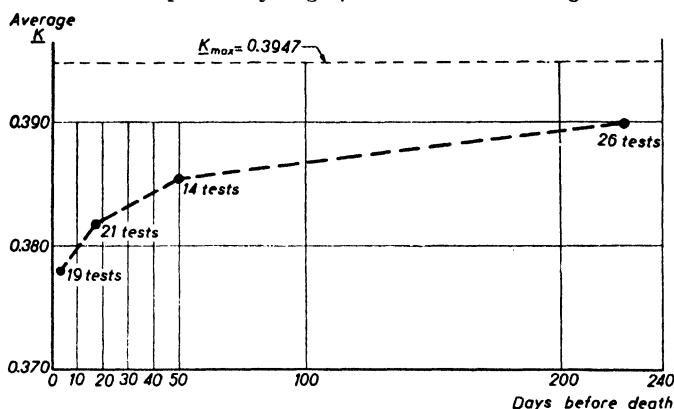


Fig. 4. Terminal fall in  $K$ , the plasma-protein equilibrium factor

against the average interval prior to death.  $K$  thus falls steadily with the approach of death (and increased clinical severity). In several instances the  $A/G$  ratio even increased prior to death (see case 1).

Also, with the exception of two cases of rheumatoid arthritis who were sufficiently ill as to require admission to hospital, no case recovered where  $K$  fell below 0.380 in the left upper and lower sections of Fig. 3. Admittedly the  $A/G$  ratio in most of these cases was also low, but in several it was as high as 1.5 which is not regarded as of fatal significance.

b. The orthogonals, drawn at right-angles to the contours, represent the paths along which a plasma must move in order to achieve the maximum change in  $K$  with the least change in the actual values of the protein fractions — just as the quickest way of descending a hill is to move at right-angles to the contours.

These orthogonals are not just fanciful insertions and attain significance when it can be shown that serial results tend to move along them during progress and recovery in some cases (see case 3).

In many instances the clinical changes could be represented equally well by the changes in the simple A/G ratio but attention is drawn to that part of Fig. 3 where A/G equals about 1.2 to 1.5, where the orthogonals are almost vertical. Here are found cases (such as case 3) in which decrease in  $K$  truly reflects the worsening of the clinical condition while the A/G ratio remains almost unchanged.

It will be realized that the orthogonals actually inserted into Fig. 3 are only a few of the possible infinite number of such lines.

c. All cases do not follow the orthogonals however, *i.e.*, the rate of change is not maximal. In these, the serial results may fall on Fig. 3 so that the  $K$  values follow regular definite trends: or the serial results may appear to fall at random on Fig. 3 yet more careful analysis shows that the  $K$  values have a regular increase or decrease numerically which correspond to the changes in the clinical condition. While the A/G ratio may increase and decrease irregularly from day to day, the introduction of the A/F ratio is such as to correct for these irregularities and produce a regular even alteration in the  $K$  values.

This again illustrates the superior reliability of  $K$  compared with the A/G ratio.

d. In some instances (as in case 2) it may be found that the ultimate clinical outcome can be indicated by the factor  $K$  before any other empirical test, such as the E.S.R. or the plasma viscosity. In case 2,  $K$  retreated from the threshold of fatal values (0.380) while the plasma viscosity was still rising.

e. The  $K$  factor is being used in search of malignant lesions in parts of the body not readily available to direct investigation. Where such a condition is suspected,  $K$  (and more especially serial  $K$  evaluation) frequently confirms its presence by indicating a reaction to organic changes in the body while other tests (E.S.R., plasma viscosity, etc.) are negative or ambiguous (see case 4). Wide infiltration by a malignant tumour may be associated with insignificant changes in  $K$  in a few cases, yet, if it is remembered that the test is non-specific and if other causes of systemic reaction can be excluded, the proportion of cases in which the estimation of  $K$  is of real assistance to the clinician makes the test worth performing.

In serial estimations, a movement of  $K$  to the left and downwards would favour a diagnosis of malignancy; and a  $K$  moving to the right and upwards would be strongly against such a diagnosis (see case 5).

#### CASE HISTORIES

Five case histories only are presented. They include most of the points made out in the general statements above. The results are summarized in Table I.

1. Case A.B.; senile patient with gangrene of feet and extensive bed sores.

This case illustrates the steady decrease in the  $K$  values as death approaches, without a corresponding consistent change in the A/G ratio.

2. Case I.R.; elderly patient with phthisis.

The  $K$  value here shows the ultimate outcome correctly and earlier than the other two tests. The  $K$  value increases, indicating a reduction in the systemic reaction to the tuberculous focus, while the plasma viscosity is still increasing. In this instance the A/G ratio would have been equally informative and the E.S.R. parallels the change in  $K$ .

3. Case B.R.; acute rheumatic fever in young man.

This case is especially instructive.

Following a sore throat, the patient developed a classical attack of rheumatic fever, for which he was admitted to hospital. Sodium salicylate was pushed for three weeks, with only a partial response, when the drug had to be discontinued on account of toxic symptoms. Calcium aspirin was given as a substitute.

Test No. 1 was performed on the day before the salicylate was stopped. The stoppage was followed by an immediate relapse in the clinical condition as indicated by elevation of temperature, increased sweating, increased joint pains, etc. This deterioration is reflected in the movement of  $K$  down an orthogonal, while there is no significant change in the A/G ratio: the plasma viscosity increase parallels the change in  $K$ , but the E.S.R. shows a definite decrease.

Three days after test No. 2 sodium salicylate treatment was resumed; two days later still, some septic teeth were removed while the patient was receiving large prophylactic doses of penicillin. Clinical improvement thereafter was steady so that he was discharged two weeks later to continue resting at home.

Tests Nos. 3, 4, and 5 show the movement of  $K$  approximately along an orthogonal while the A/G ratio shows no change. The changes in the plasma viscosity again parallel those of  $K$ , but the E.S.R. changes are more erratic.

Test No. 6 was performed at his first attendance as an out-patient. There was no evidence of cardiac involvement.

4. Case J.H.; non-detectable malignant growth.

This patient, a man of 57 years, consulted his doctor in October 1946 with a history of some months loss of weight, cough, rectal bleeding and vague abdominal pains. After a thorough investigation in hospital, including barium meal, pyelograms, etc., no organic lesion could be detected apart from bleeding piles which were dealt with surgically. There was an evening elevation of temperature. Malignancy was considered, but no primary growth could be detected. A diagnosis of Periarteritis Nodosa was also considered possible although this was not confirmed by muscle section.

He was readmitted in January 1947 on account of increasing anaemia.  $K$  was estimated at this time and its very low value indicated a severe systemic reaction to an organic change, with a fatal outcome highly probable. Thorough investigation still failed to reveal this organic lesion.

Later he was investigated at another centre where a diagnosis of Aleukaemic Leukaemia was made. However, two weeks before his death in May 1947, he suddenly developed haematuria and a swelling in the lumbar region which left no doubt as to the true diagnosis of 'Hypernephroma'.

5. Case K.S.; pathological fracture in middle-aged patient.

This case illustrates the value of serial estimation of  $K$ .

The patient was in rather poor general health; there was vomiting frequently after meals. While moving in bed, the right femur fractured. Skiagrams showed another small area of rarification in this femur, sclerosis of the other femur, and a mottling of the skull

TABLE I

Case	Test No.	Date of death	Date of test	A	G	F	A/F	A/G	K	Corrected Citrate Plasma Viscosity	Maximum Citrate E.S.R. (mm/h)
1 A.B.	1	24/3/44	13/3/44	3.64	2.14	0.91	3.99	1.70	0.380	1.837	225.0
	2		15/3/44	3.55	2.54	1.02	3.47	1.40	0.376	1.961	540.0
	3		21/3/44	3.64	2.48	1.11	3.25	1.46	0.371	2.041	420.0
2 I.R.	1	—	4/12/43	2.75	3.15	0.54	5.06	0.87	0.380	1.663	90.0
	2		31/1/44	3.17	3.35	0.38	8.31	0.95	0.386	1.720	46.8
			22/2/44	3.33	3.29	0.49	6.75	1.01	0.3882	1.738	33.2
			11/8/44	3.99	3.05	0.40	9.73	1.31	0.3936	1.715	33.3
3 B.R.	1	—	12/8/47	3.62	2.90	0.56	6.43	1.25	0.393	1.639	55.7
	2		18/8/47	3.39	2.80	0.72	4.68	1.21	0.3887	1.682	41.2
	3		25/8/47	3.65	2.37	0.66	5.54	1.54	0.3905	1.647	43.5
	4		4/9/47	3.94	2.54	0.55	7.20	1.55	0.3938	1.614	35.1
	5		11/9/47	3.81	2.48	0.47	8.14	1.54	0.3943	1.575	28.8
	6		9/1/48	4.72	1.96	0.37	12.82	2.41	0.382	1.530	7.4
4 J.H.	1	May '47	4/2/47	3.80	3.69	1.51	2.52	1.03	0.359	—	—
5 K.S.	1	—	1/4/47	3.99	2.66	0.60	6.65	1.50	0.393	1.695	48.4
	2		15/4/47	4.43	2.70	0.56	7.92	1.64	0.3942	1.664	96.0

Case 1 (A.B.) = senile with gangrene of feet and extensive bed sores

„ 2 (I.R.) = elderly patient with phthisis

„ 3 (B.R.) = acute rheumatic fever in young adult

„ 4 (J.H.) = malignant growth, non-detectable clinically

„ 5 (K.S.) = pathological fracture of femur, ? cause

which was reported as consistent with secondary malignant deposits. In addition to those shown in Table I, the following laboratory results were obtained:

TABLE II

Serum Calcium	= 16.3 mg/100 ml
Serum Inorganic Phosphate	= 3.9 mg/100 ml
Serum Acid Phosphatase	= 2.3 K.A. units/100 ml
Serum Alkaline Phosphatase	= 19.5 K.A. units/100 ml

The consensus of several opinions was that this was a case of gastric carcinoma with secondary bone deposits. Treatment was symptomatic, chiefly directed at relieving the vomiting.

The picture was changed entirely when *K* was again estimated two weeks later. *K* now indicated an improvement. An improved *K*, especially when produced mainly by an increase in the albumin fraction, was inconsistent with a diagnosis of malignancy. A revised diagnosis of a simple parathyroid adenoma was confirmed at operation.

(Support of the diagnosis of non-malignancy was given by the perfect relationship of the E.S.R. and the plasma viscosity; also, the phenomenon in the plasma viscosity which has been called "Terminal decay" by HOUSTON, HARKNESS, AND WHITTINGTON<sup>3</sup> was absent.)

## DISCUSSION

Since its introduction by VAN SLYKE, the A/G ratio has been studied in a wide variety of clinical conditions and considerable information has been gained. More recently the tendency has been to study the absolute values of the fractions and less reliance has been placed on the A/G ratio. The weakness of the A/G ratio lies in the fact that it presents only a part of the protein change which is going on in the blood and apparently the fibrinogen cannot be neglected if a true stereoscopic picture is to be obtained.

In support of the contention of the importance of the fibrinogen, it may be noted that the present writer found a poor correlation between the physical properties of serum and the clinical condition, in contrast to the excellent correlation between the clinical state and the physical properties of the plasma (HOUSTON *et al.*<sup>3</sup>; HARKNESS *et al.*<sup>4</sup>; COWAN AND HARKNESS<sup>5</sup>.)

The concept of the factor  $K$  depends on two suppositions, (a) that in the plasma there exists a combined-protein molecule which is in dynamic equilibrium with the free molecules, and (b) the changes in the plasma proteins parallel the systemic reaction to the original change.

The question of the existence of a combined-protein molecule has been and is being discussed by many workers in this field. This combined-molecule may break up very readily so that no method yet devised may estimate it although PEDERSEN<sup>6</sup> has found a combined-protein molecule, his 'X-protein', which has stood up to the strain of the ultracentrifuge although its recovery varies greatly according to the experimental conditions.

It has long been held that an organic change in the body causes a systemic reaction which includes a change in the plasma proteins, and that the degree of change in the proteins parallels the degree of systemic reaction. This is the basis of the E.S.R. test and the various flocculation reactions.

One of these empirical tests, the plasma viscosity estimation, depends entirely on changes in the proteins, the effect of gross changes in the non-protein constituents being negligible. In previous publications a close correlation has been shown to exist between the plasma viscosity (and hence the plasma proteins) and the systemic reaction and the severity of the organic changes.

It would appear to be logical to study the fundamental changes in the plasma protein fractions themselves rather than functions dependent upon these changes. There have been two obstacles to this however.

Firstly, up to the present scheme, there has been no simple method or single numerical value to indicate the changes which are occurring in all three fractions of the plasma proteins. The attempt at producing the results as a single figure in the A/G ratio was defective as it noted only part of these changes and as a result, showed poor correlation with the clinical condition.

Secondly the accurate estimation of the protein fractions is relatively more expensive in time, material and laboratory working space. When compared with the simple empirical tests, the Kjeldahl estimation is a formidable procedure, while the simpler colorimetric, turbidometric, and similar techniques do not yield sufficiently reliable results.

Both of these objections are surmounted by the present procedure wherein the

$K$  factor indicates the changes in all three fractions and our micro-Kjeldahl technique reduces the Kjeldahl distillation procedures to a total of about 40 minutes.

The factor  $K$  is related to the true 'equilibrium constant' of a hypothetical equation of equilibrium between the protein fractions. It is interesting to speculate upon the changes in  $K$  as related to SCHOENHEIMER's' ideas of constant change in the molecules and tissues of the body. There is an attraction in the idea of  $K$  first increasing as the rate of tissue change is increased to react upon the organic bodily change and then gradually falling as the primary impetus is gone. When recovery begins,  $K$  again increases, to fall finally into the lower normal levels. When the processes of the body are so overwhelmed that recovery will not be possible, the condition is reflected in the depression of  $K$  below a limiting value (0.380). The factor  $K$  and its movement in Fig. 3 assists the clinician in understanding the way in which the patient is reacting to offensive stimuli.

No attempt can yet be made to explain the mechanism which produces these changes in the plasma proteins or the organ or organs which are responsible for the changes. If it is believed that the liver forms the plasma proteins,  $K$  would appear to measure a liver function.

#### CONCLUSIONS

From the hypothesis that in plasma there exists a combined-protein molecule consisting of bound albumin-globulin-fibrinogen in equilibrium with the free portions of the albumin, globulin, and fibrinogen fractions, a factor  $K$  is derived which is related to the true 'equilibrium constant'.

A chart (Fig. 3) is presented for the calculation of  $K$  from the A/G and A/F ratios.

The factor  $K$  varies in disease and the variation is of clinical significance.

$K$  is superior to the A/G ratio as an index of the clinical condition, especially for serial investigation.

$K$  may be a more sensitive index than either the E.S.R. or the plasma viscosity.

#### Acknowledgements

Credit for the analysis which lead to the derivation of the factor  $K$  must go to Mr R. B. WHITTINGTON, M.Sc., of Manchester University.

I have to thank Dr A. A. McINTOSH NICOL, Dr R. HOUSTOUN VASEY, and Mr W. GRANT WAUGH for making available the clinical notes of cases 3, 4, and 5.

I am indebted to the Editor of *Analytica Chimica Acta* for permission to reproduce Figs 1 to 4.

#### SUMMARY

The Plasma Protein Equilibrium Factor is defined.

A chart is produced by which the factor can be evaluated when the albumin, globulin and fibrinogen fractions of the plasma protein are known.

The relation of this factor to the clinical condition is described and discussed, and some representative cases are presented in detail.

It is shown that the estimation of the factor is useful and informative to the clinician; it is a more reliable test than the classical Albumin/Globulin (A/G) ratio.

#### RÉSUMÉ

Le facteur d'équilibre des protéines du plasma est défini.

Un diagramme est établi, grâce auquel il est possible de calculer ce facteur, lorsqu'on connaît les proportions d'Albumine, de Globuline et de Fibrinogène dans les protéines du plasma.

Le facteur dépend des conditions pathologiques: quelques exemples de cette dépendance sont étudiés en détail. La détermination de ce facteur est utile au clinicien: elle donne des renseignements plus sûrs que le rapport classique Albumine/Globuline (A/G).

#### ZUSAMMENFASSUNG

Der Plasmaeiweiss-Gleichgewichtsfaktor wird definiert. Es wird eine Tabelle aufgestellt, aus der der Faktor bestimmt werden kann, wenn die Albumin-, Globulin- und Fibrinogenfraktionen des Plasmaeiweiss bekannt sind.

Die Beziehung dieses Faktors zum klinischen Zustand wird beschrieben und diskutiert; einige repräsentative Fälle werden eingehend beschrieben.

Es wird gezeigt, dass die Bestimmung dieses Faktors für den Kliniker nützlich und aufschlussreich ist; sie ist eine zuverlässigere Probe als das klassische Verhältnis Albumin/Globulin (A/G).

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THE ANEURINPYROPHOSPHATE CONTENT OF  
RED AND WHITE BLOOD CORPUSCLES IN THE RAT AND IN MAN,  
IN VARIOUS STATES OF ANEURIN PROVISION AND  
IN DISEASE\*

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This work has originated with the problem which method could serve best to detect slight aneurin deficiencies\*\* and to exclude the diagnosis: aneurin deficiency in patients with symptoms, resembling those of, but not caused by this disease.

Although it is not yet possible to correlate the tissue aneurin level with the optimum tissue function of aneurin the most reliable method would still be the determination of aneurin or better aneurinpyrophosphate in the patient's tissues. As this is also utterly impossible we are left with two possibilities, *viz.*, the investigation of urine (aneurin) or blood (aneurin, aneurinpyrophosphate (APP) or pyruvic acid).

#### 1. Urine

Most work has been done on urine. Yet many difficulties are encountered when drawing conclusions concerning the aneurin provision of the body from the aneurin content of the urine. There is no conclusive evidence that a small amount of aneurin excreted indicates the presence of a low amount of aneurin in the tissues, whether aneurin is determined in urine passed during 24 hours or in urine excreted during the night (fasting excretion). And though pronounced states of aneurin deficiency are indicated by absence of a significant aneurin excretion after a single oral dose of 5 or 10 mg of aneurin, no quantitative relation appears to exist between the excretion under these conditions and the aneurin provision of the body when the latter is adequate or only slightly below par.

For example, the aneurin excretion in urine suddenly drops to a much lower level upon switching from an adequate diet to a diet low in aneurin, but then remains prac-

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\* This work forms part of the investigations on aneurin metabolism by H. G. K. WESTENBRINK and collaborators.

\*\* We term "aneurin deficiency" a condition in which the cells of the body—as a whole or in part—do not operate as well as could be possible. We do not know the level of aneurin supply required for this optimal condition. Therefore we are forced, for the time being, to compare with normal, well-fed healthy individuals, living under the same circumstances, except for the difference in aneurin supply.

tically constant for a considerable time, though the body must become depleted of aneurin in the course of this period. MASON AND WILLIAMS<sup>1</sup>, *e.g.*, observed that after living for 5 months on a diet low in aneurin the excretion of this substance was not lower than as had been found after living on the same diet for 4 weeks only. It seems that the amount of aneurin excreted more likely indicates the aneurin content of the food ingested than the degree of saturation of the tissues.

Another difficulty in drawing conclusions concerning an individual is formed by the very large variations observed in numerous determinations in subjects living under equal nutritional conditions. MICKELSEN, CASTER, AND KEYS<sup>2</sup> prefer the determination of pyramine, a product of aneurin breakdown, as in individuals on the same diet the excretion of this substance does not show these great variations. But it has not been proved that a correlation exists between pyramine excretion and aneurin content of the tissues. Possibly also the pyramine excretion only reflects the aneurin content of the food digested previous to the determination.

## 2. Blood

Aneurin forms part of the prosthetic group of various enzymes catalysing intermediate reactions in carbohydrate metabolism. Hence it is to be expected that intermediate products of carbohydrate metabolism will accumulate in tissues and blood of aneurin-deficient animals and men. As most of these enzymes pertain to the various paths along which pyruvic acid may be metabolized many investigations have been carried out on the pyruvic acid content of the blood. As a matter of fact this appeared to be increased in aneurin deficiency but the differences are too small to make certain the diagnosis: aneurin deficiency from a few measurements of this acid only<sup>3</sup>. Moreover an increase of the bisulphite binding substances in blood was also found in other diseases (febrile diseases, hart disease, etc.<sup>4, 5</sup>).

We believe that determinations of APP in blood are to be preferred for detecting aneurin deficiencies. However, the directions given below should not be neglected. The determination of APP in blood is of little value without an accurate count of the blood cells and a study of the blood picture.

Several workers have determined total aneurin or APP in blood, but the wide range of variability makes it difficult to draw conclusions regarding an individual's aneurin provision. We do not exactly know the content of free aneurin of blood, but it must surely be very small as compared to the content of APP, the form in which aneurin is chiefly present in all animal tissues. For this reason the results of the determination of total aneurin are directly comparable with the results of APP determination. Table I gives a survey of the results obtained with several human subjects. The rather broad range in which the values lie, obtained by the same method, will at least partly be caused by variation in the number of blood corpuscles. For APP is only present in the formed elements. The significance of this point has been insufficiently realized by most workers in this field, as only few efforts have been made to establish a positive correlation between APP content and number of white and red cells of the blood. Only a rough estimate exists even of the ratio of the amounts of APP in red and white cells. The investigations of GOODHART AND SINCLAIR<sup>19</sup> and GORHAM AND ABELS<sup>20</sup> only, carried out with methods which are rather inaccurate in our opinion, have shown that the average leucocyte must contain several hundred times as much APP as the average erythrocyte.

TABLE I  
SURVEY OF SOME RESULTS OF INVESTIGATIONS REGARDING ANEURIN IN THE BLOOD OF NORMAL HUMAN SUBJECTS

Subjects	Substance determined	Method	γ per 100 ml		Authors	Remarks
			average and S.D.	range		
36 ♂ adults 10 ♀ adults	total aneurin	phycomyces	9 9	7-13 8-12	LEHMANN AND NIELSEN <sup>6</sup>	
47 ♂ and 26 ♀ healthy adults, 17-18 years old	total aneurin	phycomyces	7.4 ± 1.4	5.5-10.5	SINCLAIR <sup>7</sup>	no significant difference between ♂ and ♀
12 normal adults	total aneurin	thiochrome	7.9 ± 3.9	3-15	RITSERT <sup>8</sup>	could not detect any APP!
26 healthy ♂ adults 20-40 years old	APP	manometric	7.0 ± 2.1	4.5-12.0	GOODHART AND SINCLAIR <sup>9</sup>	
50 children (4-15 years old) without clinical aneurin deficiency	APP	manometric	7.5 ± 1.82	4-13	WORTIS, GOODHART, AND BUEDIG <sup>10</sup>	
14 adults	free aneurin total aneurin	thiochrome		0-1 3.3-7.3	DE JONGH <sup>11</sup>	
22 ♂ and 23 ♀ children (4-12 years old) in a hospital after "saturation" with aneurin	total aneurin	thiochrome	7.8 ± 1.3		BENSON, WITZBERGER, SLOBODY, AND LEWIS <sup>12</sup>	
20 ♂ adults	APP	manometric	11.2 ± 1.5	9.0-13.5	WESTENBRINK, STEYN PARVÉ, VAN DER LINDEN, AND VAN DEN BROEK <sup>13</sup>	
7 ♀ (18 determinations) and 29 ♂ (57 determinations) persons	total aneurin	thiochrome	♀ 5.60 ♂ 5.73	3.0-9.2 3.8-11.2	FRIEDEMANN AND KMECIAK <sup>14</sup>	
41 children and adults	APP	manometric	8.5 ± 1.5	6.0-12.7	OOSTERHUIS <sup>15</sup>	
38 ♂ and ♀ healthy, well nourished adults, 19-70 years old	total aneurin	phycomyces	7.9 ± 1.8	5.7-11.5	BANG <sup>16</sup>	
12 ♀ healthy adults on self-chosen diet	total aneurin	fermentation	5.2 ± 0.9	4.0-6.7	OLDHAM, DAVIS, AND ROBERTS <sup>17</sup>	
27 ♂ and ♀ healthy persons, 2-91 years old	total aneurin	thiochrome	11.6	9.2-14.1	LODI <sup>18</sup>	

The work described in this paper comprises the exact determination of APP in the erythrocytes and leucocytes of the rat and man, the study of the influence of aneurin-deficient diets on the APP contents of these cells and a study of the APP content of total blood and the separate cells in various diseases.

The platelets were not examined thoroughly. The amount of APP they contain must be very small, as this substance could not be detected in plasma obtained by centrifuging for 10 min at 3000 r.p.m., in which most of the platelets are still present. Nor did we try to separate the various kinds of leucocytes.

Hence the values given below for the leucocytes pertain to the average white cell. The same holds for the erythrocytes in different stages of development. We were only able to give a separate figure for nucleated red cells as distinct from all erythrocytes of later stages of development taken together.

After some preliminary attempts we abandoned the plan for a complete separation of the red and white corpuscles. After centrifuging the blood for a long time at a high rate the white cells form a tough mass which cannot be resuspended, so that it is impossible to count them. Therefore we proceeded to centrifuging at low rates for long periods of time. In this manner the red and white cells are not completely separated, but then two fractions could be obtained, one containing most of the erythrocytes and very few leucocytes and a second one containing many leucocytes and relatively few erythrocytes. It was possible to perform an accurate count in both fractions. These fractions were resuspended in plasma. By determining their APP content and the APP content of the total blood, and by counting the red and white cells in the obtained cell fractions and in the total blood three equations with two unknowns could now be drawn up, *viz.*:

$$\left. \begin{array}{l} \text{Total blood:} \quad a_1x + b_1y = c_1 \\ \text{Erythr. fraction:} \quad a_2x + b_2y = c_2 \\ \text{Leuc. fraction:} \quad a_3x + b_3y = c_3 \end{array} \right\} \quad (1)$$

Herein  $a_1$ ,  $a_2$  and  $a_3$  represent the number of erythrocytes, expressed in  $10^{11}$  per 100 ml ( $10^6$  per  $\mu$ l),  $b_1$ ,  $b_2$ , and  $b_3$  the number of leucocytes, expressed in  $10^{11}$  per 100 ml ( $10^6$  per  $\mu$ l) and  $c_1$ ,  $c_2$  and  $c_3$  the APP content, expressed in  $\gamma$  per 100 ml, in total blood, erythrocyte fraction and leucocyte fraction respectively.

$x$  and  $y$  are the APP contents of erythrocytes and leucocytes respectively, expressed in  $\gamma$  per  $10^{11}$  cells.

As the number of equations surpasses the number of unknowns, we cannot only determine the best values for  $x$  and  $y$ , but also their accuracy\*.

To this end the three equations must first of all be brought to equal precision. The inaccuracy of the coefficients  $a_n$  and  $b_n$  results from the errors adhering to the counting. Of these the irregular distribution of the cells in the counting chamber is the prevailing source of errors. If the counting is carried out as described below this error of distribution does not exceed that which can be theoretically calculated according to POISSON.

In this case the following applies:

$$\text{Standard deviation (S.D.)} = \sqrt{\text{number of cells counted.}}$$

The coefficients  $c_n$  were obtained by determining the APP contents according to

\* For the theoretical basis of the following calculations consult *e.g.*, E. CZUBER, *Wahrscheinlichkeitsrechnung I*. Verlag B. G. Teubner, Leipzig und Berlin, 1908, 2e Auflage, § 157 and following. We wish to thank Prof. Dr. M. G. I. MINNAERT for drawing our attention to this method.

WESTENBRINK *et al.*<sup>13</sup>. Estimations were at least performed in duplicate. The effect of an APP solution of unknown concentration is compared to that of a number of solutions of known content. From the latter values a standard curve is construed. This curve cannot always be drawn with the same measure of accuracy, so determinations of one series may be more reliable than those of another series, although this difference cannot be expressed in a figure. Experience gathered with a large number of estimations shows that the S.D. of values obtained by determination in duplicate is about 3%.

The S.D.'s of the coefficients of one equation now being known, and the provisional solutions of X and Y being simple to estimate from the set of equations, we can calculate the S.D. of each equation ( $\sigma_n$ ):

$$\sigma_n^2 = (\sigma_{a_n} X)^2 + (\sigma_{b_n} Y)^2 + \sigma_{c_n}^2$$

$\sigma_{a_n}$  being the S.D. of  $a_n$ , etc.

The three equations (1) can now be brought to equal precision by dividing the coefficients by the S.D. of the corresponding equation. We then obtain:

$$\left. \begin{aligned} A_1x + B_1y - C_1 &= 0 \\ A_2x + B_2y - C_2 &= 0 \\ A_3x + B_3y - C_3 &= 0 \end{aligned} \right\} \quad (2)$$

in which  $A_n = z_n/\sigma_n$ , etc.

From these equations the normal equations can be derived:

$$(A_1^2 + A_2^2 + A_3^2)x + (A_1B_1 + A_2B_2 + A_3B_3)y - (A_1C_1 + A_2C_2 + A_3C_3) = 0$$

$$(A_1B_1 + A_2B_2 + A_3B_3)x + (B_1^2 + B_2^2 + B_3^2)y - (B_1C_1 + B_2C_2 + B_3C_3) = 0$$

or:

$$x \sum A_n^2 + y \sum A_n B_n - \sum A_n C_n = 0$$

$$x \sum A_n B_n + y \sum B_n^2 - \sum B_n C_n = 0$$

These last two equations are solved. The solutions  $x_b$  and  $y_b$  are the best values appertaining to equations (1). When these values  $x_b$  and  $y_b$  are substituted in equations (2), the left members are generally  $\neq 0$ . Let the values of these members be  $\lambda_1$ ,  $\lambda_2$  and  $\lambda_3$  respectively. If no errors have been made in the calculation, then

$$\sum A_n \lambda_n = 0 \text{ and } \sum B_n \lambda_n = 0.$$

To estimate the accuracy of  $x_b$  and  $y_b$  we now calculate  $\mu$ ,  $\mu_x$  and  $\mu_y$ :

$$\mu = \sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}$$

$\mu_x$  and  $\mu_y$  are solved from the following two sets of equations:

$$\left\{ \begin{aligned} \mu_x^2 \sum A_n^2 + p \sum A_n B_n - 1 &= 0 & q \sum A_n^2 + \mu_y \sum A_n B_n &= 0 \\ \mu_x^2 \sum A_n B_n + p \sum B_n^2 &= 0 & q \sum A_n B_n + \mu_y \sum B_n^2 - 1 &= 0 \end{aligned} \right.$$

Now the S.D. of  $x_b$  is:  $\sigma_{x_b} = \mu \cdot \mu_x$   
and the S.D. of  $y_b$  is:  $\sigma_{y_b} = \mu \cdot \mu_y$ .

## METHODS

*Separation of red and white cells*

A suitable portion of the blood sample to be examined was centrifuged (of rat blood about 5 ml, of human blood 15 to 35 ml). Rat blood was centrifuged in a tube, 15 cm long and with a volume of about 5 ml, human blood in a constricted tube (Fig. 1).

The best separation of erythrocytes and leucocytes by centrifuging is obtained if the speed is gradually increased: *e.g.*,  $\frac{1}{2}$ -1 hour at 500 r.p.m., followed by  $\frac{1}{2}$ -1 hour at 1000 r.p.m. and finally  $\frac{1}{2}$ -1 hour at 1500 r.p.m. Sharper centrifuging harbours the risk that the leucocytes become so tightly packed that they cannot be resuspended homogeneously again.

When a constricted tube is used, the cell boundary must be situated at an adequate level in the narrow part of the tube. To achieve this the cell volume must previously be measured with the haematocrit and from this the amount of blood to be centrifuged can be calculated.

The best separation possible of red and white cells having thus been obtained, most of the plasma is drawn off with a pipette and the wide top part of the constricted tube is cut off. Close under the boundary between white and red blood cells a scratch is made on the glass and the tube is made to crack at this level with the aid of a drop of molten glass. Meanwhile the upper aperture of the tube is kept closed with a finger, so the top part of the tube can be removed with its contents. The white cells thus obtained, mixed with relatively few red cells, are transferred to a calibrated tube and diluted to a suitable volume with the corresponding plasma (leucocyte fraction). From the bottom of the lower part of the centrifuge tube a few ml of red cells are drawn off with a pipette, transferred to another calibrated tube and brought to adequate volume with the same plasma. This is the erythrocyte fraction.

In the total blood and the fractions thus obtained the number of red and white cells per unit of volume is now counted. This must be done with the greatest accuracy. To this end we used the method of counting as improved by us. The APP content of the three samples is determined with the manometric method of WESTENBRINK *et al.*<sup>13</sup>.

*Counting the cells*

The counting chamber of BÜRKER-TÜRK was used. According to the method in general use the blood is diluted and then simply drawn into the space between cover-glass and base by capillary forces. But we observed that the results obtained with one blood sample varied considerably according to whether the count was taken in places in the lattice, more or less distant from the side from which the chamber was filled. This impression, at first still vague, could be confirmed by taking microphotographs of the blood in the four corners of the lattice, as shown in Fig. 2. On the photographs such a large portion of the surface was taken for the count that the unavoidable distribution error of the cells would not be disturbing.

The differences were even larger when surfaces situated far outside the lattice were

References p. 64.

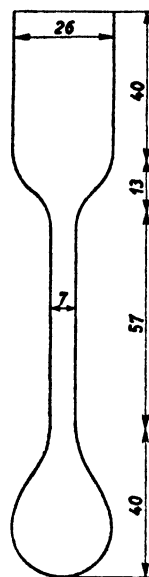


Fig. 1. Tube for the separation of erythrocytes and leucocytes in human blood (measurements in mm)

chosen for counting in this photographic manner. This is demonstrated in Fig. 3. Without exception the number of cells found in positions A and B was much higher

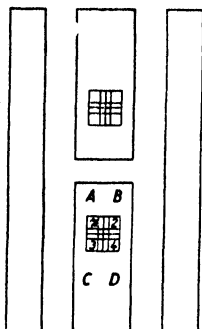


Fig. 3. Schematic drawing of the counting chamber

than that in positions C and D. Owing to some surface- or capillary effect the front of the drop that is drawn into the chamber apparently contains more cells. After having established that such a disturbing effect is very much in evidence when  $0.1 \mu\text{l}$  of blood is brought onto a slide by

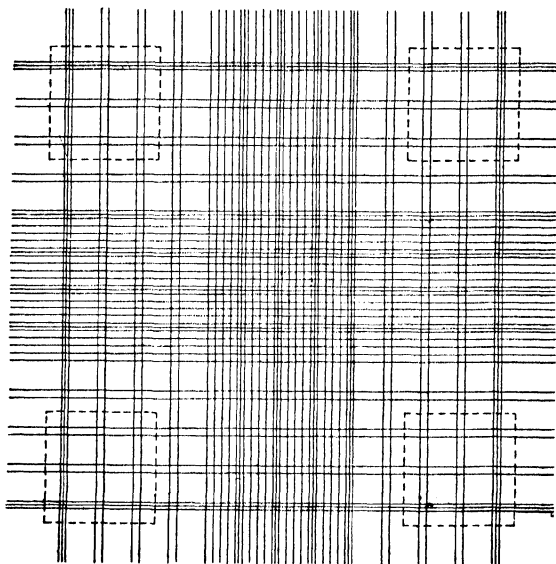


Fig. 2. The lattice in a counting chamber according to BÜRKER-TÜRK. Photographs were taken of each of the regions situated in the four corners of the lattice, as indicated by the dotted lines.

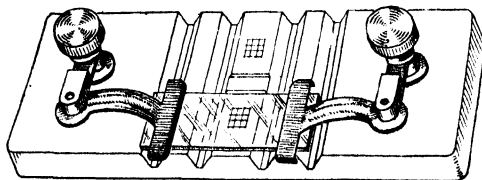


Fig. 4. Counting chamber as used when filling according to the "suction method". The cover-glass, part of a slide, covers one half of the counting chamber, the clamps press this cover-glass evenly on the chamber with the aid of a small cross beam.

area. This necessitates a small change in the clamps of the apparatus, as shown in Fig. 4.

On one side of the cover-glass the blood flows in from the pipette and on the other side it is absorbed by a slip of filter paper (see Fig. 5). When the blood has risen over a distance of 1 cm in the paper, the supply is stopped and the paper is applied a few seconds

means of a micropipette according to LINDERSTRØM-LANG and then completely counted, we arrived at the following satisfactory procedure for use with the BÜRKER-TÜRK chamber.

After diluting blood in the ordinary manner it is drawn into the counting chamber in a continuous stream. The ordinary cover-glass, covering the whole centre of the base, is exchanged for a cover-glass that only covers half of this

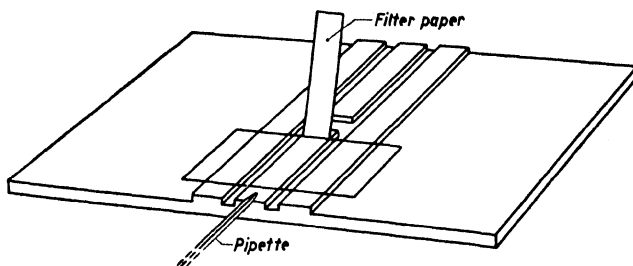


Fig. 5. Schematic diagram of the "suction method"

longer until a drop of equal size is left on both sides. Thereupon the filter paper is removed. When proceeding in this manner the cells are distributed over the whole surface according to POISSON'S Law.

Some experience is required in choosing a suitable filter paper. It should not be too "hairy" and must be neither too slow nor too rapid in absorbing the fluid. It is also important to know that absorption should not be continued until no blood is left at the other side. For then the results become irregular.

This method has been described more in detail in the *Nederland. Tijdschr. Geneesk.*<sup>21</sup>. In that paper all figures relating to the comparison of our method with the usual method for filling the counting chamber can also be found.

*Comparison of the APP content of red and white blood cells and of some organs of rats on a normal diet and of the same after receiving an aneurin-deficient diet for 5 days*

This section comprises a description of the determination of the APP content of red and white blood corpuscles of adult white rats on an adequate diet, and of rats on the same diet followed by 5, respectively 14 days on a practically aneurin-free diet. APP determinations were also carried out in liver, kidney, muscle and brain of these animals in order to see if a correlation exists between the APP content of the blood cells and that of the tissues on each of the diets.

The adequate diet was composed as follows: Two parts whole wheat flour, one part skim milk powder and 3% butter. With this diet, rich in aneurin, the rats consumed several times 10  $\gamma$  of aneurin daily.

The aneurin-deficient diet was composed as follows:

- 200 g powdered brewers' yeast, heated to 115° C for 5 hours;
- 200 g casein, freed of most of the aneurin;
- 40 g cod liver oil;
- 1400 g powdered washed polished rice;
- 100 g butter fat;
- 60 g salt mixture.

The aneurin content was about 2  $\gamma$  per 100 g, so each rat received about 0.2  $\gamma$  daily.

Under aether anaesthesia the abdominal cavity of the rat was opened and 6 to 10 ml blood could be obtained by inserting a glass canula into the aorta. This blood was collected in a tube containing about 30 mg of sodium oxalate. It was then examined in the manner described above. Meanwhile liver, kidneys, brain and leg muscle were isolated and minced with scissors on a watch-glass. About 500 mg of the brei were accurately weighed into a centrifuge tube bearing a mark at 4 ml. All determinations were carried out in duplicate. 3 ml of 0.09 N HCl were added and the mixture was boiled during one minute over a small flame, stirring continuously (the  $p_H$  should be between 2.5 and 3.0). Immediately afterwards 0.23 ml 1.16 N (6.5%) KOH were added from a microburette and the volume made up to 4 ml with distilled water. Upon centrifuging a clear extract was obtained, which was diluted with 0.1 mol phosphate buffer of  $p_H$  6.5 to a concentration, suitable for determination of APP according to the manometric method.

In Table II the results thus obtained are summarized.

In Table III we have given the correlation coefficients of the APP contents of red and white cells respectively and the contents of each of the organs examined.

By calculating the corresponding z-values (Table IV; see FISHER<sup>22</sup>, p. 198) we can decide which correlations show significant differences. In that case the difference be-



TABLE

RESULTS OF COUNTS AND DETERMINATIONS OF APP CONTENTS OF RED AND WHITE CF  
(Q = ratio of APP cont

Rat no.	Diet	Total blood			Erythrocyte fraction		
		red cells per $\mu\text{l} \cdot 10^{-6}$	white cells per $\mu\text{l} \cdot 10^{-6}$	$\gamma$ APP per 100 ml	red cells per $\mu\text{l} \cdot 10^{-6}$	white cells per $\mu\text{l} \cdot 10^{-6}$	$\gamma$ APP per 100 ml
1	Adequate diet	7.47	0.0142	28.0	6.45	0.0007	19.5
2		9.13	0.0100	20.8	6.81	0.0017	16.6
3		8.20	0.0117	17.5	7.28	0.0006	13.5
4		8.23	0.0176	21.1	6.85	0.0080	14.3
5		7.15	0.0140	19.5	6.28	0.0024	13.6
6		8.66	0.0067	21.8	7.41	0.0033	17.9
7		7.02	0.0145	15.9	5.08	0.0020	10.2
8	Aneurin-deficient diet	7.97	0.0089	8.9	8.77	0.0005	7.5
9		7.08	0.0111	12.7	6.04	0.0071	10.4
10		7.91	0.0120	7.8	8.79	0.0012	5.7
11		7.85	0.0084	9.4	9.57	0.0002	8.9
12		7.51	0.0090	10.2	10.15	0.0006	11.5
13		7.54	0.0104	10.55	9.70	0.0013	10.4
14		7.64	0.0132	11.5	9.60	0.0011	11.8
15		7.34	0.0117	10.0	9.76	0.0022	10.6
16		---	---	---	---	---	---
17		---	---	---	---	---	---

TABLE III

CORRELATION COEFFICIENTS OF APP CONTENTS OF BLOOD CELLS AND OF VARIOUS ORGANS

	Liver	Kidney	Leg-muscle	Brain
Red cells. .	0.93	0.98	0.93	0.67
White cells .	0.74	0.63	0.72	0.89

TABLE IV

Z-VALUES, CALCULATED FROM THE CORRELATION COEFFICIENTS OF TABLE III

	Liver	Kidney	Leg-muscle	Brain
Red cells. .	1.66	2.30	1.66	0.81
White cells .	0.95	0.74	0.91	1.42

tween the corresponding z-values should be at least 0.85. This holds in the following instances:

1. erythrocytes and kidney *vs.*, leucocytes and kidney;
2. erythrocytes and liver *vs.*, erythrocytes and brain;
3. erythrocytes and kidney *vs.*, erythrocytes and brain;
4. erythrocytes and muscle *vs.*, erythrocytes and brain.

TOGETHER WITH THE APP CONTENTS OF SOME ORGANS OF RATS LIVING ON VARIOUS DIETS  
(white and a red cell)

Leucocyte fraction			$\gamma$ APP per $10^{11}$ red cells		$\gamma$ APP per $10^{11}$ white cells		Q	$\gamma$ APP per gram			
red cells per $\mu\text{l.} \cdot 10^{-6}$	white cells per $\mu\text{l.} \cdot 10^{-6}$	$\gamma$ APP per 100 ml	$x_b \pm \sigma_{x_b}$		$y_b \pm \sigma_{y_b}$			liver	kidney	leg-muscle	brain
1.13	0.0274	15.5	2.94	0.035	444	7.9	151				
1.45	0.0146	11.9	2.12	0.325	588	119	277	14.2	8.0	2.8	5.4
0.95	0.0184	7.3	1.78	0.067	292	31	164	13.9	6.7	2.0	3.4
0.66	0.0128	5.0	1.80	0.105	304	32	169	11.0	6.5	2.4	3.5
0.64	0.0191	8.1	2.03	0.0012	356	0.35	175	13.9	7.4	2.3	3.6
1.24	0.0134	7.0	2.27	0.000	315	0.00	139	9.7	6.7	2.2	3.5
0.74	0.0150	4.9	1.90	0.049	222	24	117	12.2	6.7	2.8	3.6
2.26	0.0190	6.9	0.840	0.0025	246	1.4	193		3.2	1.2	3.2
0.66	0.0080	4.05	1.213	0.077	400	44	330				
1.40	0.0230	5.35	0.645	0.011	199	5.3	309	3.0	1.9	1.1	3.25
1.16	0.0208	6.2	0.931	0.0038	247	1.8	265	3.4	2.4	1.3	2.9
1.45	0.0219	7.15	1.085	0.033	252	15.9	232				
0.96	0.0243	5.9	1.076	0.041	202	13.6	188	5.2	3.1	1.65	3.2
1.13	0.0315	8.1	1.159	0.035	214	7.7	185	4.4	3.1	1.2	2.9
1.16	0.0263	6.2	1.054	0.0093	190	2.7	180	4.4	2.3	1.0	3.0
—	—	—	0.42	—	110	—	260	1.55	1.2	0.67	3.0
—	—	—	0.54	—	204	—	380	1.3	0.45	0.65	2.5

From this we may conclude that, in case of the rat, significantly closer relations, regarding APP contents, exist between red cells and kidney, as compared to white cells and kidney. Also, relations between red cells and liver, kidney or muscle are significantly closer than between red cells and brain. Or (more boldly): regarding kidney red and white cells differ principally, and regarding red cells liver, kidney and muscle differ principally from brain.

*Mean values of APP contents of red and white blood cells of rats on adequate diets and after 5 days on a diet low in aneurin*

As has already been mentioned, the accuracy of each calculated APP content of red and white cells could be ascertained. This accuracy is rather variable, so it is not correct to regard the arithmetical mean as a good statistic. Values determined with greater accuracy should bear more weight in calculating the mean than values that are less accurately established.

In Table V an example has been given of one of our calculations of the best mean.

When  $\sigma_t$  = standard deviation as a measure for the scattering of the calculated APP contents around their arithmetical mean,

$\sigma_e$  = standard deviation as a result of experimental inaccuracy,

$\sigma_{ph}$  = standard deviation as a measure for the physiological scattering,

then  $\sigma_{ph}^2 = \sigma_t^2 - \sigma_e^2$ .

$\sigma_p^2$  is calculated in column 4:  $\sigma_e^2 = \frac{\sum \sigma_{e_n}^2}{n}$ .

References p. 64.

TABLE V  
EXAMPLE OF CALCULATION OF THE BEST MEAN OF AN APP CONTENT (WHITE CELLS; RATS 5 DAYS ON ANEURIN-FREE DIET)  
(for elucidation consult the text)

I	2	3	4	5	6	7	8	9	10	11	12
Rat no.	y <sub>b</sub>	σ <sub>y<sub>b</sub></sub>	σ <sub>y<sub>b</sub></sub> <sup>2</sup>	(y <sub>b</sub> - $\bar{y}_b$ ) <sup>2</sup>	w · 10 <sup>4</sup>	w · y <sub>b</sub> · 10 <sup>4</sup>	(y <sub>b</sub> - $\bar{y}_b$ ) <sup>2</sup> · w · 10 <sup>4</sup>	(y <sub>b</sub> - $\bar{y}_b$ ) <sup>2</sup> · w · 10 <sup>4</sup>	w <sub>corr.</sub> · 10 <sup>4</sup>	w <sub>corr.</sub> · y <sub>b</sub> · 10 <sup>4</sup>	(y <sub>b</sub> - $\bar{y}_b$ ) <sup>2</sup> · w <sub>corr.</sub> · 10 <sup>4</sup>
8	246	1.4	2.0	4	2.343	576.5	70	164	2.758	678.4	229
9	400	44	1936	24336	1.613	645.2	26374	42530	1.799	719.6	47840
10	199	5.3	28.1	2025	2.329	463.5	1490	3470	2.738	545.0	3933
11	247	1.8	3.2	9	2.343	578.8	88	206	2.757	681.1	281
12	252	15.9	252.8	64	2.214	557.8	207	458	2.579	649.9	588
13	202	13.6	185	1764	2.247	453.9	1267	2847	2.625	530.4	3198
14	214	7.7	59.3	900	2.313	494.9	557	1288	2.715	581.1	1422
15	190	2.7	7.3	2916	2.341	444.8	2266	5304	2.754	523.4	6059
mean:	244		σ <sub>e</sub> <sup>2</sup> = 309.2 σ <sub>t</sub> <sup>2</sup> = 4574 σ <sub>ph</sub> <sup>2</sup> = 4265	32018	17.743	4215.4	32319	56267	20.725	4908.9	63550
						$\bar{y}_b$ = 237.6 $\bar{y}_b$ = 236.9		σ <sub>ph</sub> <sup>2</sup> = 3624			σ <sub>ph</sub> <sup>2</sup> = 3424

$\sigma_t^2$  is calculated in column 5:  $\sigma_t^2 = \frac{\sum (y_{b_n} - \bar{y}_b)^2}{n - 1}$ .

The weight of an observation  $w = \frac{1}{\sigma_{ph}^2 + \sigma_{en}^2}$  (column 6).

A corrected mean is now calculated:  $\bar{y}_{b_{corr.}} = \frac{\sum w_n y_n}{\sum w_n}$  (columns 6 and 7).

The tentative physiological scattering can now be replaced by a calculated value, considering that also the deviation of an observation from the mean does not always carry the same weight:

$$\sigma_{ph_{corr.}}^2 = \frac{\sum w_n (y_{b_n} - \bar{y}_b)^2}{\sum w_n} \cdot \frac{n}{n - 1} \text{ (column 9).}$$

Should the corrected mean deviate markedly from the tentative arithmetical mean computed in column 2, then it is desirable to regard this corrected mean as a new tentative mean and to repeat the correction in the same manner, now using the corrected physiological scattering.

This calculation may seem excessively extensive for such a limited material. Nevertheless it is not so much out of place here as with an extensive material, for in the latter case a sufficiently large number of observations falls by random within the limits of each range of accuracy.

The corrected means thus obtained are summarized in Table VI.

TABLE VI  
CORRECTED MEANS OF APP CONTENTS OF BLOOD CELLS AND TISSUES OF THE RAT  
(Q = ratio of APP contents of a white and a red cell)

Subjects	$\gamma$ APP per $10^{11}$ red cells $\pm$ S.D.	$\gamma$ APP per $10^{11}$ white cells $\pm$ S.D.	Q	$\gamma$ APP per gram			
				liver	kidney	leg-muscle	brain
Well-nourished rats	$2.1 \pm 0.41$	$340 \pm 101$	160	12.5	7.0	2.4	3.8
Rats after 5 days on aneurin-deficient ration	$1.0 \pm 0.19$	$240 \pm 58$	240	4.1	2.7	1.25	3.1
Decrease after 5 days on aneurin-deficient ra- tion	52 % t = 6.8 n = 13 $P \ll 0.01$ very significant	30 % t = 2.4 n = 13 $0.02 < P < 0.05$ significant		67 %	62 %	48 %	18 %

These results show that in the case of the well-fed rat an average leucocyte contains 160 times as much APP as an average erythrocyte. After 5 days on an aneurin-free diet the content of the red cells has decreased more than that of the white cells. In agreement herewith the ratio of the contents of a white and a red cell has risen to 240.

For the average normal rat with an average number of erythrocytes of  $7.8 \cdot 10^6$  and of leucocytes of  $0.012 \cdot 10^6$  per  $\mu$ l of blood, one will therefore find that per 100 ml of blood, containing 20.5  $\gamma$  APP, on the average 16.4  $\gamma$  or 80% is present in the erythrocytes and 4.1  $\gamma$  or 20% in the leucocytes.

TABLE VII  
APP CONTENTS OF TOTAL BLOOD, RED AND WHITE CELLS OF HEALTHY, NORMALLY FED MEN  
(Q = ratio of APP contents of a white and a red cell)

Sub- ject no.	Total blood		Erythrocyte fraction		Leucocyte fraction			Q
	red cells per $\mu\text{l} \cdot 10^{-6}$	white cells per $\mu\text{l} \cdot 10^{-6}$	$\gamma$ APP per 100 ml	red cells per $\mu\text{l} \cdot 10^{-6}$	white cells per $\mu\text{l} \cdot 10^{-6}$	$\gamma$ APP per 100 ml	$\gamma$ APP per 10 <sup>11</sup> white cells	
1	4.00	3.0	7.8	6.49	1.2	10.4	1.497	260
2	4.29	6.0	8.6	7.61	4.6	10.9	1.572	157
3	4.96	5.5	8.8	7.25	4.2	12.3	1.481	198
4	4.84	8.15	8.55	8.01	3.5	12.5	1.401	175
5	4.63	4.8	8.8	6.26	1.6	9.5	1.505	191
6	4.64	7.3	10.9	5.18	0.1	8.9	1.660	194
7	4.85	8.9	9.25	4.89	0.3	7.55	1.514	151
8	5.12	6.4	8.65	4.78	1.6	7.2	1.400	208

\* Reduced to normal cell volume if necessary.

TABLE VIII  
APP CONTENTS OF TOTAL BLOOD, RED AND WHITE CELLS OF HEALTHY, NORMALLY FED WOMEN  
(Q = ratio of APP contents of a white and a red cell)

Sub- ject no.	Total blood		Erythrocyte fraction		Leucocyte fraction			Q
	red cells per $\mu\text{l} \cdot 10^{-6}$	white cells per $\mu\text{l} \cdot 10^{-6}$	$\gamma$ APP per 100 ml	red cells per $\mu\text{l} \cdot 10^{-6}$	white cells per $\mu\text{l} \cdot 10^{-6}$	$\gamma$ APP per 100 ml	$\gamma$ APP per 10 <sup>11</sup> white cells	
1	4.15	6.6	7.6	5.75	0.1	7.8	1.38	173
2	4.20	7.8	7.45	4.74	0.2	6.05	1.203	208
3	4.32	6.1	7.3	4.83	0.2	5.7	1.188	251
4	4.35	8.0	7.7	4.10	0.1	5.3	1.16	274
5	4.53	6.0	8.6	4.79	0.2	6.3	1.354	234
6	4.76	9.3	8.65	4.60	0.2	6.7	1.375	212
7	4.18	6.7	8.55	4.92	0.2	7.2	1.382	166
8	4.48	5.5	7.0	4.87	0.2	6.1	1.24	230
9	4.04	6.2	7.2	5.31	0.2	7.3	1.36	216
10	4.06	7.9	7.2	4.85	0.4	6.75	1.227	207
11	5.20	7.2	8.6	5.04	0.1	6.5	1.45	238
12	3.93	7.3	7.4	5.14	0.2	7.3	1.25	176
13	4.71	4.5	7.0	4.64	0.0	5.6	1.211	228
14	3.90	6.5	6.5	5.02	0.1	6.9	1.258	231
							196	156

\* Reduced to normal cell volume if necessary.

*APP content of red and white blood cells of healthy human subjects on normal and aneurin-deficient diets*

Table VII gives the results of the determinations of APP in blood of 8 men and Table VIII those for 14 women, while Table IX shows the corrected means of these determinations.

TABLE IX  
CORRECTED MEANS OF APP CONTENTS OF BLOOD CELLS AND OF TOTAL BLOOD OF HEALTHY, NORMALLY FED MEN AND WOMEN

	$\gamma$ APP per $10^{11}$ red cells $\pm$ S.D.	$\gamma$ APP per $10^{11}$ white cells $\pm$ S.D.	$\bar{Q}$	$\gamma$ APP per 100 ml total blood $\pm$ S.D.
Men	$1.49 \pm 0.083$	$290 \pm 53$	195	$8.9 \pm 0.9$
Women	$1.28 \pm 0.088$	$270 \pm 34$	210	$7.6 \pm 0.7$
Difference	very significant $t = 5.5$ $n = 20$ $P < 0.001$	not significant	not significant	very significant $t = 3.8$ $n = 20$ $P < 0.001$

In the course of these determinations and of experiments with blood of patients as described below we observed cases with unusually high APP contents of the red cells. These cells appeared to have abnormally large volumes. After these observations had been made we determined the mean corpuscular volume ( $= \frac{\text{haematocrit volume (\% of total blood)}}{\text{number of erythrocytes} \times 10^{-7} \text{ per } \mu\text{l}}$ ) of each sample of blood investigated. The normal average of the mean corpuscular volume is 87. As the amount of APP present in a red cell may be expected to be proportional to the volume of the cell, all APP contents pertaining to red cells in Tables VI and VII are reduced to the average cell volume, *viz.*, the cell volume corresponding with the mean corpuscular volume 87.

The determination of the mean corpuscular volume had been omitted in the first experiments carried out with human blood. It was estimated some months later, after we had become aware of its importance. The mean corpuscular volume of these bloods deviated only very little from 87, while the mean corpuscular volume of the blood of an individual which had been found to be abnormally high some months earlier had retained this high value. Hence we did not hesitate also to include in Table VII the results obtained without simultaneous determination of the mean corpuscular volume.

The figures in Tables VII to IX demonstrate that the observed differences in APP content between the erythrocytes and between the total blood of men and women are very significant, while the difference between the leucocytes of both sexes is not significant. The contents of total blood could be expected to differ for both sexes, as their red cell counts are not the same. But the difference is larger than could be expected on these grounds, owing to the different APP content of the erythrocytes of men and women.

The standard deviation of the APP contents of the white cells is much larger than that of the red cells. This may be due to larger experimental errors in counts and APP determinations in the case of the white cells. When these larger experimental errors are accounted for\* in calculating the probability that the divergence of the standard deviations for red and white cells be due to random causes, the latter difference appears also to have a physiological cause. In other terms: there exists a significant difference between the biological variations of the APP content of white and red cells. ( $F = 2.34$ ;  $n_1 = 19$ ;  $n_2 = 19$ ;  $P < 0.05^{**}$ ).

\* For method of calculation, see example in Table V.

\*\* A. LINDER, *Statistische Methoden*, Birkhäuser, Basel, 1945, p. 57.

The larger biological variation of the APP content of the white cells may be caused by variations in the ratios of the numbers of the various forms of white cells and by differences in the age of these cells. In one individual the white cell count is subject to much larger fluctuations than the red cell count. Therefore much greater differences in the rate of cell production and so in the mean age of the white cells may be expected to occur. The possible influence of the age of the cells on their APP content is rendered probable by the observation, in blood of patients suffering from pernicious anaemia and erythroblastosis foetalis (as described below), that in an earlier stage of development red cells contain more APP than in a later stage. It seems possible that this also applies to white cells, including variations in the age of mature cells.

TABLE X

APP CONTENTS OF TOTAL BLOOD AND RED AND WHITE BLOOD CELLS OF HEALTHY INDIVIDUALS ON AN ANEURIN-DEFICIENT DIET

Subject	Days on diet	$\gamma$ APP per 100 ml total blood	$\gamma$ APP per $10^{11}$ red cells $\pm$ S.D.	$\gamma$ APP per $10^{11}$ white cells $\pm$ S.D.	Remarks
A, ♂	0	8.9	$1.53 \pm 0.060$	$280 \pm 15$	
	2	8.6	$1.50 \pm 0.046$	$292 \pm 16$	
	4	6.0	$1.21 \pm 0.106$	$214 \pm 36$	
	6	5.3	$1.06 \pm 0.173$	$156 \pm 53$	
	8	6.4	$1.16 \pm 0.055$	$180 \pm 21$	
	10	5.6	$1.13 \pm 0.022$	$139 \pm 5$	
B, ♂	0	9.0	$1.54 \pm 0.075$	$313 \pm 31$	
	5	7.6	$1.27 \pm 0.060$	$297 \pm 21$	
	10	6.9	$1.11 \pm 0.007$	$243 \pm 2$	
C, ♀	0	7.6 <sup>*</sup>	$1.38 \pm 0.065$	$239 \pm 17$	menstruation on the 4th day of the diet
	6	6.6	$1.22 \pm 0.025$	$277 \pm 7$	
	10	5.2	$1.03 \pm 0.022$	$191 \pm 6$	
D, ♂	0	8.8	$1.505 \pm 0.086$	$288 \pm 38$	on the 6th day 200 g of syrup was consumed, containing 2 mg aneurin
	4	7.5	$1.23 \pm 0.060$	$218 \pm 24$	
	10	7.1	$1.21 \pm 0.043$	$217 \pm 20$	
E, ♂	0	7.7	$1.37 \pm 0.052$	$235 \pm 13$	
	5	6.9	$1.24 \pm 0.042$	$203 \pm 12$	
	10	6.5	$1.25 \pm 0.062$	$200 \pm 20$	
F, ♂	0	10.0	$1.66^* \pm 0.062$	$323 \pm 12$	
	5	8.1	$1.24^* \pm 0.010$	$258 \pm 3$	
	10	7.8	$1.28^* \pm 0.040$	$202 \pm 9$	
G, ♂	4	8.2	$1.32 \pm 0.080$	$234 \pm 16$	
	10	6.7	$1.11 \pm 0.001$	$184 \pm 1$	

\* Reduced to normal cell volume.

Table X contains the results of determinations carried out with 7 healthy men and women, subjected to an aneurin-deficient diet consisting of: boiled or steamed polished

References p. 64.

rici, which had been carefully washed with tap water, butter fat (repeatedly melted with water), sugar, the white of 8 eggs daily, tea without milk and some condiments. Rice, butter and sugar were consumed ad libitum. This diet was followed for 10 days.

It was impossible to give a statistical evaluation of the figures in Table X as the number of individuals of equal sex is too small and the determinations were carried out at various times after the beginning of the diet. That only few individuals were subjected to this experiment was due to the fact that only people convinced of the importance of strict adherence to the diet could be trusted not to take any other food. Causes beyond our control prevented us from examining the subjects on the same day of the dietary period.

Notwithstanding these imperfections we feel justified in concluding that the APP content of red and white cells decreases rather rapidly on an aneurin-free diet. Presuming that the decrease proceeds at a constant rate we can calculate from the values found at the beginning and the end of the ten-day period (Fig. 6) that after 4.3 and 5.7 days respectively the APP content of red and white cells decreased to a value significantly lower than normal.

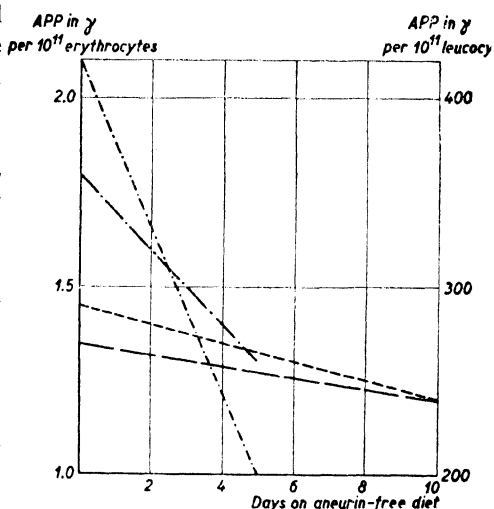


Fig. 6. Average decrease of the APP content of the blood cells of human subjects and rats on an aneurin-free diet

----- erythrocytes of human blood  
 ---- leucocytes of human blood  
 - · - · - leucocytes of rat blood  
 · · · · · erythrocytes of rat blood

*APP content of blood cells of patients suffering from various diseases characterized by abnormal corpuscular composition of the blood, and of patients suffering from aneurin deficiency*

We have investigated eight patients only, but this small number was sufficient to prove that deviations from the normal corpuscular composition of the blood cause deviations from the normal APP content of the blood, the latter bearing no relation whatsoever to the aneurin provision of the body.

TABLE XI  
PATIENT I (PERNICIOUS ANAEMIA)

	red cells per $\mu$ l total blood $\times 10^{-6}$	white cells per $\mu$ l total blood $\times 10^{-6}$	reticu- locytes	mean corpuscular volume	$\gamma$ APP per			
					100 ml total blood	$10^{11}$ red cells	$10^{11}$ white cells	$10^{11}$ red cells of normal volume
1st day in hospital . . . . .	2.45	0.0037	7 <sup>0</sup> / <sub>100</sub>	132	7.1	2.68	200	1.77
After 4 weeks in hospital, just before "Pernaemon" treatment	2.01	0.0043	12 <sup>0</sup> / <sub>100</sub>	112	6.8	2.70	300	2.10
After 5 days' "Pernaemon". .	2.32	0.0041	40 <sup>0</sup> / <sub>100</sub>	100	7.3	2.86	220	2.49



Patient I was a man suffering from pernicious anaemia, characterized by anisocytosis, poikilocytosis and polychromasia. The red cells were much larger than normal. During our investigation of this patient treatment with liver ("Pernaemon", Organon) was begun. The results are summarized in Table XI. Most workers in this field would consider the APP content of the total blood before treatment to be normal (about 7  $\gamma$  per 100 ml). Also in our opinion it lies in the normal range. Yet the number of red cells is much below the normal value. But this is compensated by the abnormally high amount of APP per red cell.

PANNEKOEK-WESTENBURG AND VAN VEEN<sup>23</sup>, OOSTERHUIS<sup>16</sup> and ROWLANDS AND WILKINSON<sup>24</sup> have also determined aneurin or APP in blood of normal persons and patients suffering from diverse anaemias. They have tried to establish a positive correlation between aneurin (APP) content and red cell count. In PANNEKOEK-WESTENBURG AND VAN VEEN's opinion part of the low aneurin values observed might be ascribed to the low red cell count in these cases of anaemia. OOSTERHUIS arrived at a similar conclusion. ROWLANDS AND WILKINSON found a difference between pernicious anaemia and anaemia caused by iron deficiency. These authors observed normal aneurin values in their pernicious anaemia patients and concluded that in these cases no correlation existed between aneurin content and red cell count. The low aneurin content in iron deficiency would be caused by a simultaneous lack of aneurin in the food. Hence in general no correlation seemed to exist between aneurin (APP) content of total blood and red cell count.

In our opinion this correlation does indeed exist. That the investigators mentioned above failed to establish this correlation has various causes:

1. they omitted to count the white cells;
2. they compared blood of healthy people to that of patients without accounting for the fact that the red cells of the latter are, as regards age and volume, not strictly comparable to the cells of normal blood.

ROWLANDS AND WILKINSON's results can be explained as follows:

In pernicious anaemia normal aneurin (APP) contents of total blood are found owing to two factors with opposite effect:

1. a number of red cells below normal;
2. a higher average amount of APP per red cell.

In the anaemia due to iron deficiency the APP content is lowered, owing to two factors with the same effect:

1. a lowered number of red cells;
2. a lowered amount of APP per red cell, due to the increased average age of the red cells or the abnormally small volume of them.

An abnormally low APP content of the blood does not necessarily indicate aneurin deficiency, but may be caused by an abnormal corpuscular composition of the blood.

Considering the figures in Table XI one observes that the APP content of total blood remains constant notwithstanding 5 days of "Pernaemon" treatment. The mean corpuscular volume had declined, but the average amount of APP per red cell did not show a corresponding decrease. Reduced to normal volume the APP content of the red cells is even increased. Liver therapy causes the apparition in the blood of a large number of young erythrocytes, as is shown in Table XI by the rise of the percentage of reticulocytes. Obviously these cells have a higher APP content than fully matured cells.

The investigation of patients II, III and IV furnished more evidence for the assumption that the APP content of the red cells decreases with increasing age.

Patient II was a woman suffering from severe haemorrhage caused by carcinoma of the liver. There was a very active regeneration of red cells; even normoblasts (5% leucocytes) were observed in peripheral blood. The results of our determinations are summarized in Table XII. The number of red cells is very low. Yet the APP content of the total blood is higher than that of the healthy women we examined. This is explained by the fact that the average amount of APP per red cell is considerably higher than normal. Also in this case the increased APP content of the red cells appears to correspond with a lower average age of these cells.

TABLE XII

PATIENT II (SEVERE HAEMORRHAGE CAUSED BY CARCINOMA OF THE LIVER)

Red cells per $\mu$ l total blood $\times 10^{-6}$	White cells per $\mu$ l total blood $\times 10^{-6}$	Mean corpuscular volume	$\gamma$ APP per			
			100 ml total blood	$10^{11}$ red cells	$10^{11}$ white cells	$10^{11}$ red cells of normal volume
1.53	0.0204	97	9.9	2.50	340	2.34

Patients III and IV were two new-born children suffering from erythroblastosis foetalis. The blood contained many reticulocytes and nucleated erythrocytes. We were able to determine the APP content of the latter by further fractional centrifuging of the red cell fraction of the blood. This content appeared to be extremely high, *viz.*, 100 to 160  $\gamma$  per  $10^{11}$  cells, while the non-nucleated red cells contained 5 to 6  $\gamma$  and the white cells 100 to 200  $\gamma$  per  $10^{11}$  cells. Even the content of the non-nucleated red cells is very high when compared to red cells of healthy adults. These observations confirm our provisory conclusion, mentioned above, that red cells in earlier stages of development or young cells contain more APP than older cells.

TABLE XIII

PATIENT V (LYMPHATIC LEUCAEMIA OR LYMPHOSARCOMA)

Red cells per $\mu$ l total blood $\times 10^{-6}$	White cells per $\mu$ l total blood $\times 10^{-6}$	Mean corpuscular volume	$\gamma$ APP per			
			100 ml total blood	$10^{11}$ red cells	$10^{11}$ white cells	$10^{11}$ red cells of normal volume
2.23	0.0089	100	5.3	1.60	200	1.40

Table XIII refers to the examination of a well-nourished man (patient V) suffering from either lymphatic leukaemia or lymphosarcoma. The figures show that the number of red cells is lowered, while the number of white cells and the APP contents of both kinds of cells are normal. The APP content of total blood, however, is lower than the lowest value observed in the healthy subjects we examined. This is merely due to the lowered number of red cells and must not be ascribed to a shortage in aneurin provision. This case proves decisively that one is not justified in concluding to aneurin deficiency from determination of APP in total blood alone.

TABLE XIV

PATIENT VI (MYELOID LEUCAEMIA)

Red cells per $\mu$ l total blood $\times 10^{-6}$	White cells per $\mu$ l total blood $\times 10^{-6}$	$\gamma$ APP per		
		100 ml total blood	$10^{11}$ red cells	$10^{11}$ white cells
1.78	0.686	218	1.3	310

Examination of patient VI, a child suffering from myeloid leukaemia, showed that normal aneurin provision can also concur with an extremely high APP content of total blood (see Table XIV). The APP content of both white and red cells was quite normal. The high APP content of total blood is solely caused by the extremely high number of white cells. This child might have suffered from severe aneurin deficiency while the APP

content of the total blood would still have been much higher than normal. This supposed aneurin deficiency would not have been detected by determination of APP in total blood alone.

The next patients were suspected to suffer from aneurin deficiency. Patient VII was a woman with pylorus stenosis. She was nourished by plasma infusions. The figures in Table XV demonstrate that the number of red cells is only slightly lowered, but that the APP content of total blood as well as that of red and white cells has decreased considerably. Hence these determinations confirm the occurrence of aneurin deficiency as was expected from the anamnesis.

TABLE XV  
PATIENT VII (PYLORUS STENOSIS)

Red cells per $\mu$ l total blood $\times 10^{-6}$	White cells per $\mu$ l total blood $\times 10^{-6}$	$\gamma$ APP per		
		100 ml total blood	$10^{11}$ red cells	$10^{11}$ white cells
3.64	0.0091	3.4	0.83	85

Patient VIII was a man suffering from tropical sprue, with the usual disturbance of intestinal absorption. Determinations were carried out before and after treatment with 2 mg aneurin daily during one month. Table XVI shows the results. The APP content of total blood, red and white cells was very low previous to the administration of aneurin and normal after treatment. The patient was obviously suffering from aneurin deficiency.

TABLE XVI  
PATIENT VIII (TROPICAL SPRUE)

	Red cells per $\mu$ l total blood $\times 10^{-6}$	White cells per $\mu$ l total blood $\times 10^{-6}$	Mean corpus- cular volume	$\gamma$ APP per			
				100 ml total blood	$10^{11}$ red cells	$10^{11}$ white cells	$10^{11}$ red cells of normal volume
Before treatment	4.23	0.0080	102	3.2	0.62	80	0.53
After treatment with aneurin	3.10	0.0096	1.06	8.0	1.65	275	1.36

The general conclusion from our examination of these patients is that it is possible to detect aneurin deficiency by determination of APP in blood, but that this must be combined with accurate study of the corpuscular composition of the blood. Separate determination in both red and white cells is to be preferred to determination in total blood, but even then the haematological examination should not be omitted.

#### SUMMARY

1. Methods are described for accurate counting of red and white blood cells and for the determination of the aneurinpyrophosphate (APP) content of these cells.

2. These methods were applied to: a) rat blood (adequately nourished rats and rats on an aneurin-deficient diet); b) human blood (healthy subjects on their usual diet or on an aneurin-deficient diet, and patients). Moreover APP was determined in liver, kidney, brain and leg-muscle of the rats.

3. The mean values of the APP contents of the blood cells and the tissues of the adequately nourished rats were: red cells: 2.1  $\gamma$  per  $10^{11}$  cells; white cells: 340  $\gamma$  per  $10^{11}$  cells; liver: 12.5  $\gamma$  per g;

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kidney: 7.0  $\gamma$  per g; brain: 3.8  $\gamma$  per g; leg-muscle: 2.4  $\gamma$  per g. For rats after 5 days on an aneurin-free diet these values were: red cells: 1.0  $\gamma$  per  $10^{11}$  cells; white cells: 240  $\gamma$  per  $10^{11}$  cells; liver: 4.1  $\gamma$  per g; kidney: 2.7  $\gamma$  per g; brain: 3.1  $\gamma$  per g; leg-muscle: 1.25  $\gamma$  per g. In the well-fed rat an average leucocyte contains 160 times as much APP as an average erythrocyte. After 5 days without aneurin the ratio of the contents of a white and a red cell has risen to 240. So on an aneurin-free diet the content of the red cells decreases more rapidly than the content of the white cells.

4. The red blood cells of the man have a significantly higher APP content than the red cells of the woman. No significant difference was found between the respective contents of the white cells. The average values were: man: 1.49  $\gamma$  per  $10^{11}$  red cells, 290  $\gamma$  per  $10^{11}$  white cells; woman: 1.28  $\gamma$  per  $10^{11}$  red cells, 270  $\gamma$  per  $10^{11}$  white cells. The ratio of the contents of a white and a red cell is about 200.

5. In men on an aneurin-free diet the APP content of red cells decreases after 5 days to a value significantly lower than normal. White cells appear to lose their APP at approximately the same rate.

6. Red cells in earlier stages of development, as occurring in the blood of some anaemia patients, contain higher amounts of APP than normal red cells. This is also the case for red cells with an abnormally large volume. As a consequence of the abnormal APP contents of the blood of anaemia patients abnormal APP contents of total blood can occur, bearing no relation to the aneurin provision of the body. Therefore a haematological examination should be combined with each APP determination in blood aimed at the detection of a possible aneurin deficiency. The determination of APP in red and white cells is to be preferred to the determination in total blood. Examples are given in which the occurrence of aneurin deficiency could be proved to exist by working along these lines.

## RÉSUMÉ

1. Description de méthodes pour la numération précise des globules rouges et des globules blancs et pour le dosage de la teneur en pyrophosphate d'aneurine (APP) de ces cellules.

2. Ces méthodes ont été appliquées à: a) du sang de rat (rats soumis à un régime complet et rats soumis à un régime carencé en aneurine); b) du sang humain (individus normaux soumis à leur régime habituel ou à un régime carencé en aneurine, et individus en état pathologique). En outre, l'APP a été dosée dans le foie, le rein, le cerveau et la musculature des pattes des rats.

3. Les valeurs moyennes des teneurs en APP des éléments du sang et des tissus des rats soumis à un régime complet sont les suivantes: globules rouges: 2.1  $\gamma$  par  $10^{11}$  cellules; globules blancs: 340  $\gamma$  par  $10^{11}$  cellules; foie: 12.5  $\gamma$  par g; rein: 7.0  $\gamma$  par g; cerveau: 3.8  $\gamma$  par g; musculature de la patte: 2.4  $\gamma$  par g. Chez les rats soumis pendant 5 jours à un régime carencé en aneurine, on a trouvé: globules rouges: 1.0  $\gamma$  par  $10^{11}$  cellules; globules blancs: 240  $\gamma$  par  $10^{11}$  cellules; foie: 4.1  $\gamma$  par g; rein: 2.7  $\gamma$  par g; cerveau: 3.1  $\gamma$  par g; musculature de la patte: 1.25  $\gamma$  par g. Chez le rat soumis à un régime complet, un leucocyte moyen contient 160 fois plus de APP qu'un érythrocyte moyen. Après 5 jours de carence en aneurine, le rapport des teneurs d'un globule blanc et d'un globule rouge monte à 240. Ainsi, chez les animaux soumis à un régime carencé en aneurine, la teneur des globules rouges décroît plus rapidement que celle des globules blancs.

4. Les globules rouges du sang de l'homme ont une teneur en APP nettement supérieure à celle des globules rouges de la femme. Aucune différence nette n'a été trouvée en ce qui concerne les teneurs respectives en APP des globules blancs. Les valeurs moyennes sont: homme: 1.49  $\gamma$  par  $10^{11}$  globules rouges, 290  $\gamma$  par  $10^{11}$  globules blancs; femme: 1.28  $\gamma$  par  $10^{11}$  globules rouges, 270  $\gamma$  par  $10^{11}$  globules blancs. Le rapport des teneurs d'un globule blanc et d'un globule rouge est environ 200.

5. Chez des hommes soumis à un régime carencé en aneurine, la teneur en APP des globules rouges décroît après 5 jours d'une façon nette. Les globules blancs perdent leur APP approximativement à la même vitesse.

6. Les globules rouges dans leur premier stade de développement, tels qu'on les rencontre dans le sang de quelques malades souffrant d'anémie, contiennent des quantités de APP supérieures à la teneur normale des globules rouges. Le même phénomène se retrouve chez les globules rouges anormalement gros. Il en résulte que chez les malades souffrant d'anémie, on peut rencontrer des teneurs anormales du sang total en APP, qui n'ont rien à voir avec la réserve en aneurine de l'organisme. Aussi, conviendrait-il de combiner un examen hématologique avec chaque dosage de l'APP dans le sang fait en vue de déceler une carence éventuelle en aneurine. On doit préférer un dosage de l'APP dans les globules rouges et les globules blancs à un dosage dans le sang total. Des exemples sont donnés montrant qu'il est possible de caractériser des carences en aneurine par cette méthode.

## ZUSAMMENFASSUNG

1. Methoden zur genauen Zählung der roten und weissen Blutkörperchen und zur Bestimmung des Aneurinpyrophosphat (APP)-Gehaltes dieser Zellen werden beschrieben.

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2. Die Methoden wurden auf: a) Rattenblut (Ratten auf vollständigem Futter und Ratten auf aneurin armer Diät); b) Menschenblut (gesunde Personen auf ihrer gewöhnlichen Diät oder auf aneurin armer Diät, und Patienten) angewandt. Ausserdem wurde APP in der Leber, Niere, dem Gehirn und dem Beinmuskel der Ratten bestimmt.

3. Die Durchschnittswerte des APP-Gehaltes der Blutkörperchen und Gewebe der Ratten auf vollständiger Nahrung betrugen: rote Blutkörperchen:  $2.1 \gamma$  pro  $10^{11}$  Zellen; weisse Blutkörperchen:  $340 \gamma$  pro  $10^{11}$  Zellen; Leber:  $12.5 \gamma$  pro g; Niere:  $7.0 \gamma$  pro g; Gehirn:  $3.8 \gamma$  pro g; Beinmuskel:  $2.4 \gamma$  pro g. Bei Ratten, die fünf Tage aneurinfrei ernährt waren, betrugen diese Werte: rote Blutkörperchen:  $1.0 \gamma$  pro  $10^{11}$  Zellen; weisse Blutkörperchen:  $240 \gamma$  pro  $10^{11}$  Zellen; Leber:  $4.1 \gamma$  pro g; Niere:  $2.7 \gamma$  pro g; Gehirn:  $3.1 \gamma$  pro g; Beinmuskel:  $1.25 \gamma$  pro g. Bei gutgefütterten Ratten enthält ein Leukozyt im Durchschnitt ungefähr 160 mal soviel APP wie ein Erythrozyt. Nach fünf Tagen ohne Aneurin steigt das Verhältnis des Gehaltes eines weissen und roten Blutkörperchens auf 240. Bei aneurinfreier Diät nimmt also der Gehalt der roten Blutkörperchen schneller ab als der der weissen.

4. Die roten Blutkörperchen des Mannes haben einen bedeutend höheren APP-Gehalt als die der Frau, während bei den weissen Blutkörperchen kein signifikanter Unterschied gefunden wurde. Die Durchschnittswerte betrugen: Mann: rote Blutkörperchen:  $1.49 \gamma$  pro  $10^{11}$  Zellen, weisse Blutkörperchen:  $290 \gamma$  pro  $10^{11}$  Zellen; Frau: rote Blutkörperchen:  $1.28 \gamma$  pro  $10^{11}$  Zellen, weisse Blutkörperchen:  $270 \gamma$  pro  $10^{11}$  Zellen. Das Verhältnis des Gehalts eines weissen und roten Blutkörperchens beträgt ungefähr 200.

5. Beim Menschen sinkt nach fünf Tagen ohne Aneurin der APP-Gehalt der roten Blutkörperchen auf einen Betrag, der stark unter dem normalen liegt. Weisse Blutkörperchen scheinen ihr APP ungefähr in demselben Mass zu verlieren.

6. Rote Blutkörperchen in frühen Entwicklungsstadien, wie sie im Blut mancher Anämiepatienten vorkommen, enthalten höhere APP-Beträge als normale rote Blutkörperchen. Dies ist auch bei Zellen mit abnormal grossem Volumen der Fall. Als Folge der — was die Blutkörperchen betrifft — abnormalen Zusammensetzung des Bluts von Anämiepatienten können abnormale APP-Gehalte des Gesamtbluts vorkommen, die in keiner Beziehung zur Aneurinversorgung des Körpers stehen. Darum sollte mit jeder APP-Bestimmung im Blut, die auf die Entdeckung eines eventuellen Aneurinmangels gerichtet ist, eine hämatologische Untersuchung verbunden werden. Die APP-Bestimmung in den roten und weissen Blutkörperchen ist der Bestimmung im Gesamtblut vorzuziehen. Es werden Beispiele gegeben, bei denen das Auftreten eines Aneurinmangels durch Bestimmungen nach diesen Richtlinien bewiesen werden konnte.

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## THE EFFECT OF CYANATE ON THE STABILITY OF PROTEINS

by

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MILLINGTON AND SCHÜTZ<sup>1</sup> recently found that the diuretic action of cyanate, as previously described by SCHÜTZ<sup>2</sup> and BIRCH AND SCHÜTZ<sup>3</sup>, was abolished when cyanate was incubated with serum. This may have been due to an enzyme capable of destroying cyanate. This question is being investigated and will be discussed separately.

An alternative explanation for the loss of biological activity of cyanate on incubation with serum would be the binding of the former by serum proteins. A reaction of this kind seems probable because of the known great reactivity of cyanate, *e.g.*, with amino acids<sup>4</sup>, methaemoglobin<sup>5</sup>, <sup>6</sup> and cytochrome *a*<sub>3</sub><sup>7</sup>.

This paper describes an effect of cyanate, consisting in stabilizing proteins against a number of denaturing agents or procedures.

## HEAT COAGULATION

*Methods and materials*

Blood was taken from human volunteers, and the serum obtained in the usual way. Samples of serum were kept at 4° for not longer than 1–2 days. Before use it was centrifuged.

**Serum proteins.** A purified fraction of serum albumin was prepared as follows. Solid ammonium sulphate (250 g/litre) was added to fresh horse serum, and the precipitate rejected. A further 150 g/litre of ammonium sulphate were added to the supernatant and the precipitate dissolved in the smallest possible quantity of water. After dialysis against distilled water at 4° the lipoids of this solution were extracted by precipitating the proteins in ethanol below –15°, according to HEWITT'S<sup>8</sup> modification of HARDY AND GARDINER'S method. The ether washed precipitate was then extracted in a Soxhlet for 48 h with ether containing metallic sodium, both of which were once renewed. The extracted material, a white powder, was dried *in vacuo* until the sample no longer smelled of ether. The powder was then made up into a paste with water and dialysed at 4° for several days against frequent changes of distilled water. A precipitate of insoluble material was centrifuged off and rejected before use.

**Sodium cyanate** was prepared from urea according to BADER, DUPRÉ, AND SCHÜTZ<sup>9</sup>. Only freshly prepared solutions were used. Since higher concentrated solutions of sodium cyanate were very alkaline (pH 8.0–9.0), the pH was brought to that of serum in equilibrium with air (pH 7.8–8.0), by the addition of a few drops of acetic acid or primary potassium phosphate. Equivalent amounts of the acid or potassium phosphate were added to the controls, using sodium bicarbonate instead of cyanate.

**Ammonium cyanate** was prepared as follows. A solution of sodium cyanate was precipitated by silver nitrate, the precipitate washed with cold water and acetone. A solution of ammonium chloride in equimolecular amounts was added to the dry powder, and the suspension shaken in a WARBURG shaker for 30 min at room temperature. The filtered solution was used at once to avoid greater losses through isomerization into urea.

**Caffeine** was recrystallized from alcohol. It was dissolved in serum or the protein solution, or used as caffeine citras, B.P.

**pH.** The pH of the solutions which were added to serum samples, was adjusted to that of serum. Where that was not possible, the pH nearest to 7.8 was aimed at. Usually the small amounts of substances to be tested did not greatly alter the pH of the serum. When, however, this was altered, a similar sample of serum was brought to the same pH by addition of small amounts of acetic or citric acid, of phosphate buffer solutions, or of sodium bicarbonate, and used as control.

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The urease solution consisted of a freshly made, centrifuged and filtered, aqueous extract of benzene extracted jack bean meal.

**Bilateral nephrectomy.** Both kidneys of rabbits were removed under ether anaesthesia by the usual dorsal approach. After the operation the animals were given no food, but plenty of water to drink. The animals usually survived 3 to 5 days, sometimes 7 days, if no food was given during the first 24 h after the operation. Later they spontaneously abstained from food. Blood, taken by cardiac puncture, was left 1 h at room temperature. The clot was then detached from the glass wall, left standing for further 1–3 h at room temperature, and centrifuged. The serum was again centrifuged after separation from the clot, and used within 4 h.

**Addition of substances to serum.** Whenever possible the substances to be tested for their action on proteins, were added to the serum or protein solutions, in solution, except when very high concentrations of caffeine and cyanate were added. In this case a part of the added quantities was dissolved directly in the serum. Urea was always added in solution; in order to prevent higher concentrations of urea from developing locally, the liquid was kept agitated while the urea solution was added drop by drop.

**Determination of heat coagulation temperature.** A number of procedures have been used to determine the effect of heat on serum proteins<sup>10, 11, 12</sup>. While some of these methods are undoubtedly of great sensitivity, they either are lengthy procedures, or not independent of individual factors of the observers.

The simple method described below, was developed solely for the purpose of this investigation. Although it is probably less accurate than some of the known methods, it allowed rapid and fairly accurate comparisons of the effect of different substances on samples of the same batch of serum. The method is independent of individual factors of different observers, and gave repeatable results. One determination took *circa* 15 min.

The principle of the method was to record the sudden change of heat conduction when the serum coagulated. The device is shown in Fig. 1. A test tube (A) containing serum, or serum protein solution,

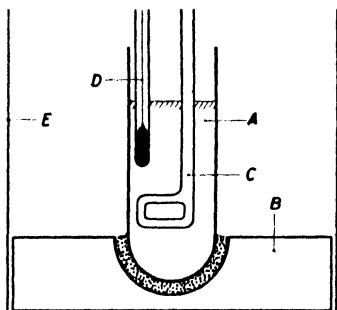


Fig. 1. Device used for the determination of coagulation-temperature and -time. The protein solution in test tube A is heated by the hot plate B, contact being made through sand. C = stirrer. D = thermometer. E = circular draught excluder. When coagulation occurs, the heat conduction from B towards the thermometer is reduced, causing a sudden fall in temperature, as shown by the heating curves (Figs. 2 and 4).

was placed in a groove of an electric heater (B). The heating coil was covered by a cement layer. A little sand, placed in the groove, assured uniform contact. The heated area of the test tube did not vary substantially in different experiments. A motor driven mixer of the glass ring type (C) and a thermometer (D) were placed into the test tube as shown in Fig. 1. Care was taken to immerse the thermometer to equal depths in all experiments. The mixer was lifted 1 cm high, and fell to the bottom of the tube by its own weight, 140 times/min. A circular draught excluder (E) was placed around the lower part of the test tube. The test tube was covered with loosely packed cotton wool. The heating was arranged to bring the contents of the test tube from room temperature to 70° in *circa* 8–9 min. The heating current was kept constant by means of a resistance. The rate of heating varied very little. The current, for the heating and the mixer, was switched on when the serum was filled in and the arrangement was rigidly set up.

Temperature readings were taken at intervals of 15 sec and plotted against time. From a typical curve, shown in Fig. 2 it can be seen that the temperature suddenly fell, when coagulation occurred.

While the serum remained liquid, the heat supplied from the bottom of the tube, was evenly distributed by the mixer. When coagulation took place, the conduction of heat towards the thermometer was much decreased; thus the heat loss into the surrounding air from the thermometer was not any more made good by heat conduction from the heated area upwards. Hence the sudden fall of temperature.

To obtain the effect, the heating should be confined to one area only, and an appreciable heat loss should take place from the remainder of the surface. When heated in a waterbath no drop of temperature occurred. The drop of temperature seemed simultaneous with the sudden increase in viscosity. It was found best to place the thermometer near the wall, which caused a more rapid and sudden fall of temperature when coagulation occurred. The lower end of the thermometer was at a distance of 1.6 cm from the bottom of the tube. The same thick walled test tube was used for all experiments. 3.6 ml serum were added to the tube, and 0.2 ml, if not otherwise mentioned, of the various solutions tested for their activity. In control experiments a similar volume, containing equimolecular amounts of sodium chloride, or of a buffer solution, was added.

The period from the time when the serum reached 20°, till coagulation occurred, was read from

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the heating curve (Fig. 2). Obviously coagulation takes place at lower temperatures when the heating is prolonged, and vice versa. In Fig. 3 the temperature, when coagulation occurred, is plotted against the time needed to heat different samples from 20° until coagulation occurred. It can be seen that normal human sera showed small variations in this respect.

By addition to serum, or serum proteins, of a substance capable of stabilizing proteins against heat, both the temperature of coagulation is raised, and the time of heating lengthened. Obviously it was desirable to express the results with one figure only. Trials to heat at a constant temperature and record only the time needed for coagulation, gave such huge differences of coagulation times, due to the very great temperature coefficient of heat denaturation, that the above described method seemed more advantageous. *E.g.*, a serum sample without addition took 22 min to coagulate at 70.9°. Additions of cyanate increased the time to many hours or days, when heated at the same temperature. With the above described method, however, a result was obtained within 20 min. Moreover, the time needed to raise the temperature of the samples from 20° to the coagulation temperature ("heating time"), did not vary greatly, usually less than  $\pm 20\%$ .

By applying the following simple correction it was found possible to express the results contained in this paper with one figure only. The accuracy was sufficient for our purpose.

By extrapolating the rising and falling parts of the heating curve (see Fig. 2) a crossing point is obtained, indicating the coagulation temperature, and the heating time. *E.g.*, a serum sample coagulated at 72.8° with a heating time of 9 min 15 sec. On addition of the substance to be tested the same serum coagulated at 77.4°, with a heating time of 11 min 45 sec. Once it was assured that the coagulation temperature and heating time of the untreated sample corresponded well with the values usually obtained with normal sera (Fig. 3), the temperature corresponding to the heating time of 11 min 45 sec was obtained from the standard curve (Fig. 3), thus: 71.4°. The difference between this temperature and the temperature, when coagulation actually occurred (77.4°), is 6.0°, which, in this paper, would represent the result, *i.e.*, the rise of the heat coagulation temperature corrected

for time of heating. If not otherwise mentioned, this correction was applied to the data of heat coagulation temperatures given in this paper.

When several determinations of the coagulation temperature were made on different serum samples the results showed a maximum variation of  $\pm 0.35^\circ$  from the standard curve (Fig. 3). The standard deviation from the graphically obtained mean for different sera was  $\pm 0.18^\circ$  ( $n = 11$ ), with an extreme range  $\pm 1.3^\circ$  ( $n = 11$ ).

Only such sera were used for tests, which normally gave coagulation temperatures within the limits shown by a dotted lines in Fig. 3. This was the case with most sera. It enabled the application of the correction for the time of heating to be applied by means of the standard curve, and made the construction of a new curve for each serum unnecessary.

#### EXPERIMENTAL

From typical results shown in Table I, it can be seen that cyanate was very potent in stabilizing proteins against heat coagulation.

With high concentrations of cyanate no coagulation occurred at all, even when the temperature reached boiling point. This result may, at least partly, be due to the

References p. 81.

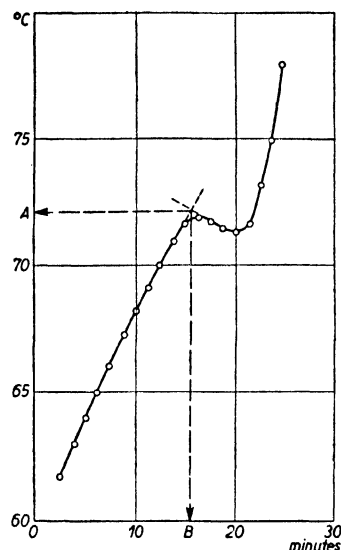


Fig. 2. Heating curve of serum. The serum was heated as shown in Fig. 1. Temperature readings were made every 15 sec. When coagulation occurred, a sudden fall of temperature was recorded. The coagulation-temperature (*A*) and coagulation-time (*B*) were obtained from the curve, after extrapolation of the rising and falling parts of the heating curve.

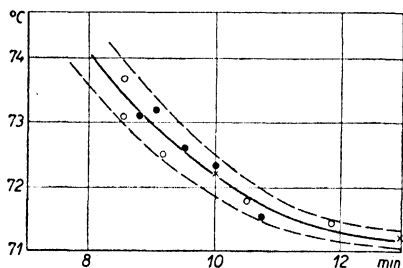


Fig. 3. Relation of the coagulation temperature to the time of heating needed to produce coagulation. Normal human sera.



TABLE I  
THE EFFECT OF THE ADDITION OF DIFFERENT SUBSTANCES ON THE  
HEAT COAGULATION OF HUMAN SERUM AND SERUM ALBUMIN

Final molar concentration in serum	Rise of heat coagulation temperature (corrected for time of heating), above that observed with a sample of the same serum, or protein solution, without addition °C
Sodium cyanate, 0.043	1.6, 1.8, 1.0, 2.2, 1.1
" " 0.086	3.4, 4.0, 4.2, 5.0
" " 0.166	8.0, 7.1, 8.2
" " 0.191	no coagulation, 13.5
Potassium thiocyanate, 0.040	± 0
" " 0.086	1.5, 1.3
" " 0.166	2.8, 3.2
" " 0.191	4.1, 4.6
Urea, 0.16	± 0
" 0.19	± 0
" 1.0	± 0
" 3.0	2.2
" 4.0	2.8
" 6.0	no coagulation
Sodium cyanide, 0.16	± 0
Potassium ferricyanide, 0.16	1.8, 1.1
Urethane, 0.11	± 0
Pilocarpine, 0.10	1.8, 1.8
Picrotoxin, 0.08	± 0
Strychnine hydrochloride, 0.1	± 0
Caffeine, 0.10	2.8
" 0.12	3.1, 3.5
Theobromine sodium acetate, 0.1	± 0, ± 0
Theophylline sodium acetate, 0.1	± 0, ± 0
Sodium salicylate, 0.1	2.5, 3.2
Final concentration in a purified fraction of serum albumin	
Sodium cyanate, 0.04	1.6, 2.0, 2.1
" " 0.086	3.0, 3.1

fact that on heating a solution containing relatively large amounts of cyanate, the reaction soon becomes alkaline, unless the solution is very strongly buffered. Alkali is, of course, also capable of preventing heat coagulation of serum. When the  $p_H$  was controlled by adding a highly concentrated buffer solution, a very marked difference in the coagulation temperature of the sample and control was still observed. The addition of concentrated buffer tended to lower the heat coagulation temperature of the controls.

On several occasions the  $p_H$  of the clot was measured with a glass electrode, after the clot was cooled to room temperature. With final concentrations of cyanate in serum up to 0.04 *M*, the  $p_H$  was not much altered in comparison with the control, both being in the range of  $p_H$  8.0. The  $p_H$  of serum containing higher concentrations of cyanate was higher, (0.2–0.8 units), than that of the control, although strongly buffered. When, however, a similar  $p_H$  change was produced in controls, through addition of sodium

bicarbonate, the coagulation temperature was not as much affected as through cyanate. It must be concluded that the observed effect was due to cyanate, and not to a change of  $p_H$ .

Comparing the action of different substances on heat coagulation, no substance was found quite as active as cyanate at or near the usual  $p_H$  of serum, when the latter was in equilibrium with air ( $p_H$  7.8–8.0). Caffeine and salicylate seemed nearly as active, thiocyanate, though still active, was much less potent than cyanate, salicylate and caffeine. Ferricyanide seemed slightly active, while cyanide had no influence at all (Table I).

Many effects of cyanate were found to be similar to those of caffeine. Not only have both substances a diuretic action<sup>2, 3</sup>, but they are also strikingly similar in their reaction with haemoglobin derivatives. J. KEILIN<sup>13, 14</sup> drew attention to a group of effects of caffeine on a number of haemoglobin derivatives. This "caffeine effect" was characterized by the dispersion and solution, and by preventing the precipitation and aggregation of these pigments, and by the reinforcement and shift of the absorption bands. BADER, DIRNHUBER, AND SCHÜTZ<sup>7</sup> recently observed that cyanate was also capable of producing all these effects.

The action of cyanate on heat coagulation described above was therefore compared with the action of a number of other substances on heat coagulation, which were either found by J. KEILIN to produce the "caffeine effect", or were closely related to substances capable of producing this effect.

It seems remarkable, as can be seen from Table I, that caffeine raised the heat coagulation temperature nearly as much as cyanate, but that substances closely related to caffeine, like theobromine and theophylline, did not show any obvious activity in this respect. The only other substance found active in this regard, though much less than caffeine and cyanate, was pilocarpine, which was found by J. KEILIN also to show the "caffeine effect". Picrotoxin, strychnine, urethane, and uric acid had no effect whatsoever in the range of concentrations in which cyanate was very active.

Essentially the same effects were obtained with ammonium cyanate as with the sodium salt. Since, even at room temperature, appreciable amounts of the ammonium salt undergo isomerization into urea, the slightly less pronounced effects obtained with this salt are probably due to the fact that the solutions of ammonium cyanate, when used, are already less concentrated with regard to cyanate.

*Aspect of the coagulum.* When serum, with an addition of cyanate, was heated until coagulation occurred, the clot was of a very different appearance than that obtained from the control. While the coagulum of the control was firm, and somewhat brittle, that obtained from the same serum containing cyanate was more fluid and jelly-like than that of the control. It was, of course, more viscous than normal serum. In the sample containing cyanate the change in viscosity seemed to precede slightly the change in colour and turbidity. The sample containing cyanate appeared more yellow and almost transparent after coagulation, while the controls were greyish-white and quite turbid.

#### PREVENTION OF HEAT DENATURATION BY CYANATE

The effect of cyanate in raising the heat coagulation temperatures of proteins could be due to cyanate being capable (1) of preventing heat denaturation, or (2) of preventing the coagulation only of heat denatured protein, or (3) of redissolving heat coagulated

protein. The following experiment was made to obtain information regarding the first of the above mentioned possibilities.

A solution of *M*-sodium cyanate, or *M*-sodium chloride respectively, was added to two samples of the same batch of horse serum (1:9 v/v). Since the addition of sodium cyanate caused a slight shift of the  $p_H$ , the serum sample containing sodium chloride was brought to the same  $p_H$  by careful addition of *N* NaOH (final  $p_H$  of both samples was 8.0–8.2). After standing for circa 1 h at room temperature, the 2 samples were immersed simultaneously into a water bath at 72°. Already after 3–4 min the control, containing sodium chloride, became very turbid and slightly more viscous. When the samples were removed after 8 min incubation, the control was nearly completely clotted, while the sample containing cyanate was quite fluid and transparent.

In order to ascertain whether and how much undenatured protein was left in the sample containing cyanate, the following procedure was adopted. Both samples were cooled in a freezing mixture and then brought to room temperature. Solid ammonium sulphate was added until saturation, the precipitate was filtered off and washed with a saturated solution of ammonium sulphate.

While the precipitate thus obtained from the control did not dissolve in water, appreciable amounts of the precipitate obtained from the sample containing cyanate proved to be readily soluble in water. The precipitates were suspended in water and, after thorough mixing, centrifuged and filtered. A clear supernatant liquid was obtained from the sample containing cyanate. The redissolved protein present in this solution could be re-precipitated with ammonium sulphate, and redissolved in water in the usual way.

The amount of redissolved protein was determined by precipitation with trichloroacetic acid; the precipitate was centrifuged, twice washed with distilled water and dried at 105°. Unheated samples of serum, whether containing cyanate or chloride, showed the same amount of re-soluble protein (5.420 g dry weight/100 ml) after saturation with ammonium sulphate. 0.996 g/100 ml was obtained from heated serum containing cyanate, while no re-soluble protein was obtained from the heated control, containing sodium chloride.

Thus 18.4% of the amount of protein present was apparently protected from heat denaturation in the sample containing cyanate. Essentially the same results were obtained with solutions of purified fractions of serum albumin. It is now being investigated whether the "protected" protein in these heated solutions can be re-crystallized in the usual way, and whether its crystalline habit and other properties are identical with those of non-denatured protein.

#### UREA AND HEAT COAGULATION OF SERUM

##### EXPERIMENTS WITH SERA FROM ANIMALS AFTER BILATERAL NEPHRECTOMY

The addition of urea to serum had no effect whatsoever on the heat coagulation of the latter, in concentrations equimolar to those in which cyanate was highly active. It can be seen in Table I that *circa* 80–100 times the amount of urea was needed to produce an effect similar to that of cyanate.

It should be recalled that on heating aqueous urea solutions, considerable amounts of cyanate are formed through isomerization from urea. The rate of formation of cyanate greatly increases with temperature. Thus *circa* 5% of the urea present in a 0.1 *M*-solu-

tion, are transformed into ammonium cyanate at 100° within 30 min<sup>15</sup>. At lower temperatures the equilibrium is attained much more slowly, *e.g.*, at 38° it is reached only in several days, when *circa* 0.8% of the urea present in a 0.1 *M*-solution are transformed<sup>16</sup>.

When serum, containing an excess of urea, is heated, the formation of appreciable amounts of cyanate must, therefore, be expected. Our method of determining the coagulation temperature had an advantage in this respect, because the samples were heated for very short periods only. The time, during which the samples were at temperatures above 60°, was only of the order of 3–4 min. Nevertheless, at these temperatures, cyanate is formed fairly rapidly from urea. As mentioned above, *circa* 80–100 times the amount of urea was needed to produce the same effect as cyanate, with regard to heat coagulation. This ratio would correspond very well with the percentage of the amount of urea initially present which could be expected to be transformed into cyanate.

Thus a considerable part of the action of urea on heat coagulation is most probably due to the cyanate formed from this substance during the heating, and not to urea itself, unless it could be shown that the isomeric transformation urea → cyanate was completely suppressed in serum. An indication to the contrary was found, as will be shown below.

It appears that the question of exactly how much of the effect of higher concentrations of urea on the heat coagulation of serum, was due to urea itself, and how much to cyanate formed from the latter, would have to be studied separately, after establishing the kinetics of the reactions involved.

#### *Heat coagulation of sera with urea content raised in vivo*

Since the isomeric transformation of small amounts of urea into ammonium cyanate under physiological conditions, must be considered as highly probable<sup>17, 18, 19</sup>, all states characterized by a raised concentration of urea in the body, become of interest in connection with cyanate. The possibility that cyanate may play a role in the intoxication of uraemia is discussed elsewhere<sup>20</sup>. The marked influence of cyanate on the heat coagulation temperature of serum was thought to be a possible tool to help to decide whether a reverse WÖHLER reaction occurred under physiological conditions. The heat coagulation temperatures of serum and plasma of normal rabbits was therefore compared with that of serum samples obtained from the same rabbits after bilateral nephrectomy, when a high urea concentration had developed.

On all occasions the coagulation temperature of serum or plasma obtained after bilateral nephrectomy was very markedly raised (Table II), if the blood was taken not earlier than 2 days after the operation.

The results would seem in harmony with the assumption that more cyanate is formed when the concentration of urea is maintained at a raised level over relatively long periods of time. The results cannot be ascribed to artificial formation of cyanate from urea, produced by the heat applied during the determinations of the coagulation temperature, as discussed above; the urea content was raised to 10–15 times the normal concentration. Even if as much as 1% of the urea present had undergone artificial isomerization during the short heating period of the coagulation experiment, which seems extremely unlikely, this could not account for the very considerably raised heat coagulation temperatures observed after bilateral nephrectomy. If the raised coagulation temperatures were due to cyanate, the bulk of it must have been formed *in vivo*.

TABLE II

HEAT COAGULATION TEMPERATURES, AND TIMES OF HEATING NEEDED TO PRODUCE COAGULATION OF PLASMA FROM RABBITS, BEFORE AND AFTER BILATERAL NEPHRECTOMY

	Coagulation temp. ° C	Time of heating
Rabbit 1		
before bilateral nephrectomy . . . . .	80.15	10' 15"
2 days after bilateral nephrectomy . . . . .	84.5	12' 30"
3 days after bilateral nephrectomy . . . . .	85.5	13' —"
Rabbit 2		
before bilateral nephrectomy . . . . .	79.4	9' 45"
1 day after bilateral nephrectomy . . . . .	80.1	10' 15"
2 days after bilateral nephrectomy . . . . .	82.4	12' 12"
3 days after bilateral nephrectomy . . . . .	85.8	13' 45"
Rabbit 3		
before bilateral nephrectomy . . . . .	80.9	10' —"
1 day after bilateral nephrectomy . . . . .	80.6	11' —"
2 days after bilateral nephrectomy . . . . .	83.5	12' 45"
Normal rabbit 4 . . . . .	79.8	10' 15"
Normal rabbit 5 . . . . .	81.2	11' 15"

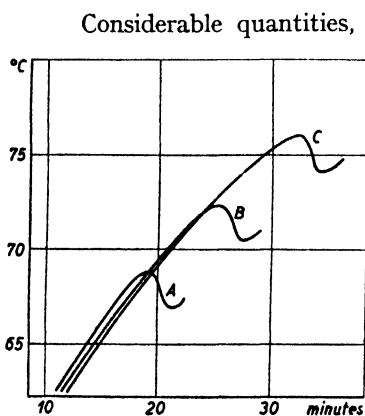


Fig. 4. Heating curves of a sample of serum; undiluted (A), and after dilution with 0.9% NaCl; B = serum: NaCl solution = 10:2, C = 10:5.

Considerable quantities, however, of many other substances are retained after bilateral nephrectomy, many of which may affect the heat coagulation temperature of serum. Moreover, bilateral nephrectomy is also regularly followed by a very pronounced dilution of the blood (hydraemia), which, as can be seen from Fig. 4, is capable of markedly raising the heat coagulation temperature of serum. It cannot therefore be decided whether the results obtained after bilateral nephrectomy can be ascribed to an excess formation of cyanate from urea, although the results do not contradict such an assumption.

A striking indication, however, that the isomeric transformation of small amounts of urea into ammonium cyanate can proceed in serum at physiological temperature and  $p_H$ , was obtained by the following experiment.

#### *Heat coagulation of sera incubated with an excess of urea*

The effect of cyanate on heat coagulation of serum made it possible to investigate whether urea can undergo isomeric transformation into ammonium cyanate in serum under physiological conditions. Since it is known that, in pure aqueous solutions of urea, isomerization takes place at an increasing rate with rising temperature, and is practically at a standstill at 4°, an excess of urea was added to a sample of serum, and one half was immediately cooled to 4°, while the other half was incubated at 38°. The time needed to attain heat coagulation at constant temperature was determined after 40 h.

Since at 4° no isomerization could be expected to occur, any delay of heat coagula-

tion or rise of the coagulation temperature of the sample incubated at 38° could be regarded as a strong indication for the formation of cyanate from urea, provided that ordinary serum, with its usual low urea content, would show no, or less, difference between the two parts, previously incubated at 4° and 38° respectively. This was indeed found in several experiments to be the case.

Since it was essential in these experiments to compare the heat coagulation of the two corresponding samples under exactly equal conditions, the determination of the coagulation time at constant temperature was found more suitable. Because of the very high temperature coefficient of heat denaturation the differences in time of samples of different behaviour towards heat, are then very great.

Urea was added, in a freshly made solution, to horse serum at room temperature, establishing a final concentration of 0.08 *M*, in excess of the amounts of urea naturally present in the serum. Immediately after mixing the urea solution with the serum, the sample was halved. One part was immediately cooled to 4° in an ice water bath, and thereafter kept in the refrigerator at an average temperature of 4°. The other half was incubated at 38° for the same period of time (40 h). Chloroform was added to the samples, as a bacteriostatic. On other occasions benzoic acid was added in sufficient amounts, which, beside acting as a bacteriostatic, brought the  $p_H$  of the serum to 7.4. The results were essentially the same, when the  $p_H$  of the 38°-sample was brought to 7.4, by keeping the serum in equilibrium with a gas phase of oxygen, containing 5% CO<sub>2</sub>.

After incubation at 4° and 38° respectively, the samples, *circa* 15 ml, were placed in stoppered boiling tubes, bound together by rubber-bands. These pairs were immersed into a water bath at 70.8° ( $\pm 0.05^\circ$ ). Provision was made to tilt the samples gently from time to time without removing them from the waterbath. This was done slowly, since brisk movements are known to influence coagulation. By tilting, the increase in viscosity and the final coagulation was observed. A light source was placed behind the tubes, so that also the turbidity was observed. An effective stirrer in the water bath, and continuous agitation of the solutions for the first 2 min assured that both halves rapidly reached the temperature of the water bath.

Two parts of a sample of horse serum, *without* any further addition of urea, previously incubated at 4° and 38° respectively, showed practically no difference on being heated at 70.8°. If anything, the sample previously incubated at 38° became sooner opalescent than that previously kept at 4°, but the difference was small, and they coagulated at practically the same time (19.5 min). The final aspect of the clot of both halves was the same.

To another batch of serum, sodium chloride, to a final concentration of 0.08 *M* was added, in excess of the amounts naturally present. Again one half was kept 40 h at 4° and 38° respectively. On heating at 70.8°, no marked difference was observed. The sample previously incubated at 38°, coagulated even slightly earlier than that previously kept at 4° (16.0 and 16.5 min). The clots of both parts looked alike.

When, however, an excess amount of urea was added to serum, and two batches subsequently incubated at 4° and 38° respectively, very great differences on heating the samples together at 70.8° were seen. A typical experiment is described below.

The sample kept at 4° became strongly opalescent after 9 min at 70.8°, and was clotted completely after 15 min, while the other half, previously incubated at 38°, was, even after 15 min heating, less opalescent than the 4°-half was after 9 min only. While the 4°-sample was coagulated after 15 min, the 38°-sample was then still quite fluid.

After 17 min the difference was still marked; after 19 min, though still significant, the difference was smaller. Only after 22 min the 38°-sample coagulated.

There was a difference of at least 7 min (32%) between the coagulation times of the two samples. The difference is significant, since ordinary serum samples without an excess of urea, previously incubated at 4° and 38°, never showed an obvious difference. Any difference which could be observed was in the opposite direction, *i.e.*, the 38°-sample coagulated slightly earlier than the 4°-sample. This difference, however, was < 6% of the coagulation time (3 experiments).

The significance of the result is, moreover, strongly supported by the observation that the clot obtained with the 38°-sample of the serum, containing an excess of urea, was always less firm and far more transparent than that of the same serum-urea mixture, previously kept at 4°. Thus also in this respect, *the 38°-sample showed all the characteristics of serum to which cyanate was added before heating.*

It should be noted that urea, in the range of concentrations used in the above described experiments, did not influence the heat coagulation of serum directly (see Table I). On prolonged incubation, however, at 38° and at  $p_H$  7.4, a very marked rise of the heat coagulation temperature was obtained, whereas no marked influence on the heat coagulation of the samples similarly incubated at 4° and 38° was obtained, when the urea content was not raised before.

It was shown, therefore, that neither urea alone, nor the incubation at 38° was the reason for the altered stability of serum towards heat. Since, however, an excess of urea *and* incubation produced this change, it is most probable that appreciable amounts of cyanate were formed from urea on incubation in serum, with all the consequent characteristics of enhanced stability towards heat.

#### SOLUBILITY OF COAGULATED SERUM PROTEINS IN SOLUTIONS OF CYANATE

10 ml portions of serum from the same batch were heat coagulated by immersion for 15 min in a boiling water bath. After cooling, the contents were broken up, cut, and ground with sand. This material was suspended in equimolar solutions of cyanate, or sodium chloride respectively. The controls, in sodium chloride solutions, were adjusted to the same, slightly more alkaline  $p_H$  of the sodium cyanate solutions. The samples, in conical flasks, were then shaken in a *Warburg* apparatus at room temperature for 3 h, centrifuged, and the supernatant liquid filtered through a retentive filter (Whatman No. 5). The nitrogen content in the liquid was then determined (*micro-Kjeldahl*.)

Already after centrifugation it was quite obvious that some of the precipitate had dissolved in the more concentrated solutions of cyanate, because they frothed. It can be seen in Table III that cyanate was capable of dissolving appreciable amounts of coagulated protein. Urea, in the same range of concentrations, was quite inactive in this respect.

#### THE STABILIZING EFFECT OF CYANATE AGAINST VARIOUS PRECIPITATING AGENTS

Cyanate was found to have a stabilizing effect against a variety of precipitating agents. In Tables IV and V the effect of cyanate against the precipitating action of alcohol and mercuric chloride is shown. There was a range of concentrations at which

TABLE III

SOLUTION OF HEAT DENATURED AND COAGULATED SERUM PROTEINS AFTER SHAKING PARTICLES IN SOLUTIONS OF SODIUM CYANATE, UREA AND SODIUM CHLORIDE, AT THE SAME pH AND AT ROOM TEMPERATURE (3 h)

The amounts of dissolved protein are expressed as N (*micro-Kjeldahl*), found in excess of the N-content in the solution due to the addition of cyanate, of urea.

Solvent	Dissolved $\mu\text{g N}/10\text{ ml}$
Sodium cyanate, 0.05 M	380
" " 0.1 M	595
" " 0.2 M	910
Sodium chloride, 0.05 M	26
" " 0.1 M	12
" " 0.2 M	18
Urea, 0.05 M	45
" 0.1 M	40
" 0.2 M	62

cyanate prevented or diminished the precipitating effect of these agents. No similar effect of cyanate was found on trichloroacetic acid precipitation, if the amount of acid neutralized by the decomposition of cyanate was taken into account. The  $p_H$  of the controls was adjusted to that of the samples containing cyanate, which caused a slight shift towards the alkaline side. A similar protecting effect of cyanate against urea and surface denaturation will be described separately<sup>23</sup>.

A typical experiment with alcohol was carried out as follows. To three portions of the same batch of horse serum, sodium chloride, sodium cyanate, or ammonium carbonate were added respectively, to give a final concentration of 0.1 M. The solutions were added in 1.0 M concentrations, in the proportion of 1:9 (v/v) to the serum samples,

TABLE IV

THE PROTECTING EFFECT OF CYANATE AGAINST PRECIPITATION OF SERUM PROTEINS BY ETHYL ALCOHOL

Serum A = containing 0.1 M-sodium cyanate; final concentration in the mixture: 0.071 M. Serum B = containing 0.1 M-sodium chloride, adjusted to the same  $p_H$  as serum A, by addition of 0.1 N NaOH

	1	2	3	4	5	6	7
0.9% NaCl, ml . . . . .	0.9	0.7	0.5	0.4	0.3	0.2	0.1
96% ethanol, ml . . . . .	0.7	0.9	1.1	1.2	1.3	1.4	1.5
Serum A, ml . . . . .				4			
Precipitation . . . . .	---	---	---	---	+	++	+++
Serum B, ml . . . . .				4			
Precipitation 1. . . . .	---	+	++	++	+++	+++	+++
Final conc. of ethanol, %	12	15	19	21	23	25	27



TABLE V

THE PROTECTING EFFECT OF CYANATE AGAINST PRECIPITATION OF SERUM PROTEINS (HORSE) BY MERCURIC CHLORIDE

Serum A = containing 0.1 M-sodium cyanate

Serum B = containing 0.1 M-sodium chloride, adjusted to the same pH as serum A, by addition 0.1 N NaOH

	1	2	3	4	5	6	7	8
HgCl <sub>2</sub> , 2% . . . . .	1.0	0.5	0.25	—	—	—	—	—
HgCl <sub>2</sub> , 1% . . . . .	—	—	—	0.25	0.12	—	—	—
HgCl <sub>2</sub> , 0.5% . . . . .	—	—	—	—	—	0.12	0.06	—
NaCl, 0.9% . . . . .	—	0.5	0.75	0.75	0.88	0.88	0.94	1.0
Serum A, ml . . . . .	1							
Precipitation . . . . .	+++	++	+	±	—	—	—	—
Serum B, ml . . . . .	1							
Precipitation . . . . .	+++	+++	++	++	+	±	±	—

and left standing for several h. 10.7 ml ethyl alcohol was then added to 10 ml of each sample. The precipitate (*p*) was centrifuged off. The supernatant (*s*) was filtered and saturated with ammonium sulphate which brought down a very much greater quantity of precipitate (*ps*) in the sample containing cyanate. These precipitates (*ps*) were then each suspended in 10 ml water to determine how much could be redissolved. After thorough mixing, the samples were centrifuged and the supernatant filtered. The amount of dissolved protein in this supernatant was then determined by precipitation with trichloroacetic acid, centrifugation of the precipitate, twice washing the latter with water, and drying it at 105°. Thus, 16.7 mg/10 ml serum dry weight was obtained from the sodium chloride sample, while 175.0 mg/10 ml was obtained from the sample containing sodium cyanate.

Since the sample containing sodium cyanate was slightly more alkaline, than the control, containing sodium chloride (0.1 pH unit), a further control solution was used, as mentioned above, by adding an equivalent amount of ammonium carbonate to serum before alcohol precipitation. This sample was even more alkaline (pH 8.6) than that containing cyanate (pH 7.8–8.0). 111 mg/10 ml of resolvable protein was obtained from this sample. This was significantly less than what was obtained from the sample containing sodium cyanate.

The precipitate (*p*), obtained immediately after the addition of alcohol to the serum samples, was apparently of much greater volume in the sample containing sodium cyanate, than in both the chloride or ammonium carbonate controls. When water was added to these precipitates the amounts of resolvable protein recovered from them were practically equal in the three cases. The sample containing sodium chloride gave 30 mg/10 ml dry weight resolvable protein, that containing ammonium carbonate 31.6 mg/10 ml, while that containing sodium cyanate gave 36 mg/10 ml.

## DISCUSSION

*The cyanate effect*

The known effects of caffeine in preventing precipitation and aggregation, promoting solution and dispersion of haemoglobin derivatives, as well as reinforcement and shift of absorption bands, are reactions, which were described by J. KEILIN and discussed as the "caffeine effect". Most of these effects can also readily be produced by cyanate instead of caffeine<sup>7</sup>. The above described experiments show that also with regard to the effect on heat coagulation of proteins, cyanate and caffeine behave similarly. The two near relatives of caffeine, theobromine and theophylline, which are, as discovered by J. KEILIN, unable to produce the caffeine effect, were also found in the above described experiments to have no effect on heat coagulation. Moreover, both caffeine and cyanate are diuretics. Both caffeine<sup>21, 22</sup> and cyanate<sup>23</sup> increase the ultra-filtration rate of dilute, buffered protein solutions. Both, as mentioned below in greater detail, have anti-mitotic activity.

Since thus, beside the "caffeine effect", cyanate shares with caffeine this remarkable number of pharmacological and physico-chemical properties, it seems likely that the underlying mechanisms of these effects are intimately related. Since strong indications were recently found for the formation of small amounts of cyanate from urea (see above, and <sup>16, 17, 18, 19</sup>), the above mentioned effects most probably have physiological significance.

*Counteraction of heat denaturation*

It has apparently not been pointed out before that the effect of a substance in raising the temperature of heat coagulation of protein solutions, often goes parallel with the effectiveness of the same substance to dissolve coagulated protein. Salicylate and urea, in high concentrations, were both found capable of raising the temperature of heat coagulation and are also known to promote solution of denatured and precipitated proteins (see ANSON<sup>24</sup>).

Because of this parallelism, it would seem conceivable that the effect of raising the temperature of coagulation was merely due to the prevention of the visible change *i.e.*, of coagulation, by redissolving the protein while, or immediately after coagulation; cyanate would thus not hinder heat denaturation, but the precipitation of heat denatured protein only. An interpretation of this kind seems very unlikely since apparently undenatured protein could be precipitated with ammonium sulphate from a heated protein solution, and a significant part of the precipitate proved readily soluble in water. This experiment suggests that, to a considerable extent, denaturation itself was prevented by the addition of cyanate. The delay of heat coagulation, or rise of the coagulation temperature, cannot only be ascribed, therefore, to a mere inhibition of coagulation or precipitation of heat denatured protein.

Since blood pigments were also found to be protected by cyanate against precipitation and aggregation, it seems probable that this effect is intimately connected with the caffeine effect. Because of the change in the absorption spectra a combination of the pigments with cyanate can be assumed. Similarly it becomes probable that cyanate also combined with proteins in forming new compounds, and that the newly formed protein-cyanate complex showed the increased stability towards denaturing agents.

The stabilizing effect on proteins of cyanate against solutions of heavy metal salts,

is probably partly due to a formation of heavy metal complexes with cyanate. It seems, however, improbable that this could account for the whole stabilizing effect in these cases, since the effect is also produced by cyanate against a variety of other agents or procedures (alcohol, heat, surface denaturation, etc.), when no combination of cyanate with some of the precipitating agents could be assumed.

#### *Mechanism of solution of coagulated protein by urea and cyanate*

It was pointed out above that approximately 80–100 times the amount of urea, than that of cyanate, was needed to achieve similar effects on the heat coagulation of proteins. The possibility was discussed that urea itself may not be directly responsible at all for the observed effect, and that this may have been due to cyanate formed from urea at the high temperature necessary to promote heat coagulation. The other observation, namely the well known fact that higher concentrations of urea are capable of redissolving denatured protein, cannot be ascribed to a formation of cyanate during the experiment, since this dissolving property of urea can be observed at relatively low temperatures, at which the formation of cyanate from urea is negligible.

The underlying mechanisms of dissolving coagulated protein in solutions of urea or cyanate respectively, seem to differ fundamentally. Practically no denatured protein is dissolved in a urea solution, except when the latter is very highly concentrated. A huge excess of molecules of urea seems to be needed for one molecule of denatured protein to go into solution, or to prevent it from aggregating with the remainder of denatured protein present in the solution.

Cyanate seems to act differently, since very much smaller concentrations are active in dissolving coagulated protein. With increasing concentration of cyanate more protein is dissolved, suggesting a stoichiometric reaction, whereas the dissolving effect of high concentrations of urea appears to be due rather to a radical change of the physical nature of the solvent.

#### *The possible physiological significance of cyanate*

The above described experiment, in which, the heat coagulation of serum was significantly raised after incubation with a small amount of urea, strongly suggests that a reverse WÖHLER reaction can indeed take place in serum at the physiological temperature and  $p_H$ . DIRNHUBER AND SCHÜTZ<sup>18, 19</sup> recently found evidence for the presence of small amounts of cyanate in incubated brain brei suspensions. Both these findings make it appear highly probable that cyanate is normally formed in the mammalian organism, and that, therefore, the above mentioned effects of cyanate concerning the stability of proteins have physiological significance.

It becomes of interest in this connection that, as was pointed out by J. KEILIN, native proteins also have a pronounced dispersing effect on certain blood pigments, an effect, which is also followed by reinforcement and shift of the absorption bands. It may thus be said that proteins exhibit the "cyanate", or J. KEILIN's "caffeine-effect", since these effects are practically identical. After having discovered that globin and serum proteins had effects on porphyrin<sup>25, 26</sup>, similar to those of caffeine on these tetrapyrrolic compounds, J. KEILIN carefully investigated whether any of a great number of the known amino acid contents of these proteins were capable of producing the "caffeine-effect". None of these amino acids was found capable of producing this effect.

One of the possible explanations offered by J. KEILIN for this finding, was that "proteins may contain an additional, not yet isolated constituent".

Since the effects of cyanate are so similar to those of proteins in this respect, it seems conceivable that cyanate may indeed be the "not yet isolated constituent", predicted by J. KEILIN. There are, of course, a number of other possible explanations for the similarity of action of proteins, cyanate and caffeine in this respect. Since, however, the formation of cyanate in the warm blooded organism from the ubiquitous urea must now be regarded as very probable, the above mentioned possibility of small amounts of cyanate being normally present in solutions of native proteins, or as natural constituents of these proteins, must be taken into consideration.

*The influence of cyanate on protein denaturation and mitosis*

Following the finding of the weak hypnotic action of cyanate in rats<sup>3</sup>, and the observation that it stopped normal growth of cats and rats<sup>1</sup>, it was suggested to Professor HADDOW and Dr SEXTON, that cyanate may have an antimitotic action, especially in view of the remarkable discovery of these authors that urethane effectively reduced the number of leucocytes in leucaemia<sup>27</sup>. Thereupon P. DUSTIN<sup>28</sup>, in HADDOW's laboratory, discovered that cyanate had indeed a potent antimitotic action.

It seems of interest in this connection that in the above described experiments urethane was not capable of raising the heat coagulation temperature of proteins, nor is it known to cause any of the other physico-chemical effects with protein solutions, which are so marked with cyanate and caffeine. Since caffeine is also an antimitotic substance, the results reported in this paper suggest that the underlying mechanisms of the antimitotic action of cyanate and caffeine on the one hand, and of urethane on the other, may be different. This would be under the assumption that the reactions with proteins of these substances are connected with their antimitotic activity.

That a connection of this kind exists is somewhat supported by the fact that thiocyanate was also found to have an antimitotic action, though this was less powerful than that of cyanate<sup>28</sup>. Thiocyanate also showed an effect on heat coagulation of proteins; again it was less potent than cyanate, also in this respect. The antimitotic activity of a number of substances, and their stabilizing effect on proteins, thus seem to show a certain parallelism.

RAPKINE<sup>29, 30, 31</sup> discussed mitosis in connection with an assumed denaturation of proteins *in vivo*, with a consequent unmasking of SH-groups. It seems of interest in this connection that cyanate has 1) a pronounced antimitotic activity, 2) effectively counteracts denaturation, and 3) combines with amino- and SH-groups<sup>4</sup>. The possible existence of an equilibrium between native and denatured protein *in vivo*, has been discussed by JOHNSON *et al.*<sup>32</sup>. If further work should finally establish the existence of an equilibrium of this kind in the warm blooded mammalian organism, cyanate would, most probably, be involved.

The fact that the relatively large number of physico-chemical and pharmacological effects mentioned above could all be produced by so simple an organic compound as cyanate, suggests that the underlying mechanisms of these effects may be intimately related. Thus, the antimitotic, hypnotic, diuretic, etc., actions of cyanate may ultimately be found to be connected with the effects of cyanate on proteins described in this paper.

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tation of the results contained in this paper, to the MEDICAL RESEARCH COUNCIL for a grant to one of us (F.S.) in aid of equipment, and to the BOARD OF MENTAL DISEASE RESEARCH for financial assistance. We are also indebted to Mr P. DIRNHUBER for much valuable help in a number of experiments.

#### SUMMARY

1. The heat coagulation temperature of serum and protein solutions was determined by means of heating curves.
2. Cyanate was found effective in stabilizing proteins to various extents against heat, heavy metal salts, and alcohol. It was more potent in some of these respects than caffeine, salicylate, thiocyanate, etc.
3. Small amounts of urea had no effect on heat coagulation, but on incubation in serum at 38° the heat coagulation temperature rose, suggesting that isomerization of urea into cyanate took place in serum at the physiological temperature and pH.
4. Cyanate protected a part of the proteins from becoming irreversibly insoluble through heat or alcohol.
5. Cyanate was found capable of dissolving denatured and precipitated protein.
6. The effect in raising the heat coagulation temperature of proteins, produced by a number of substances, seems to go parallel with the potency of these substances to promote solution of denatured protein.
7. In view of the probable formation of cyanate from urea in the organism, the heat coagulation temperature of sera with raised urea content was studied.
8. The possible physiological significance of the results, and their bearing on the antimitotic of cyanate, is discussed.

#### RÉSUMÉ

1. La température de coagulation par la chaleur du sérum et de solutions de protéines a été déterminée au moyen de courbes de chauffage.
2. Le cyanate s'est montré efficace pour protéger les protéines vis-à-vis de la chaleur, des sels de métaux lourds et de l'alcool. Son efficacité est souvent supérieure à ce point de vue, à celle de la caféine, du salicylate, du thiocyanate, etc.
3. De petites quantités d'urée n'ont aucune action sur la température de coagulation; mais après maintien dans du sérum à 38°, l'urée provoque une élévation de la température de coagulation, ce qui semble indiquer que dans les conditions de température et de pH physiologiques, l'urée s'est transformée en cyanate dans le sérum.
4. Le cyanate protège une partie des protéines dissoutes contre la dénaturation irréversible par la chaleur ou l'alcool.
5. Le cyanate provoque la solubilisation des protéines dénaturées et précipitées.
6. L'élévation de la température de coagulation par la chaleur des protéines, produite par différentes substances, semble aller de pair avec l'aptitude de ces substances à solubiliser les protéines dénaturées.
7. Du fait de la formation probable de cyanate à partir de l'urée chez les organismes, la température de coagulation par la chaleur du sérum a été étudiée en fonction de sa teneur en urée.
8. La signification physiologique possible de ces résultats est discutée, de même que leurs relations avec l'action antimitotique du cyanate.

#### ZUSAMMENFASSUNG

1. Die Temperatur der Hitzeagulation von Serum und Eiweisslösungen wurde mit Hilfe von Erhitzungskurven bestimmt.
2. Es wurde festgestellt, dass Cyanat auf Eiweiss eine stabilisierende Wirkung in verschiedenem Ausmass gegen Hitze, Schwermetallsalze und Alkohol ausübte. In verschiedenen dieser Fälle hatte es eine stärkere Wirkung als Kaffein, Salicylat, Rhodanid usw.
3. Kleine Harnstoffmengen hatten keine Wirkung auf die Hitzeagulation, aber nach Inkubation in Serum bei 38° stieg die Temperatur der Hitzeagulation, was darauf hinweist, dass in Serum bei physiologischem pH und Temperatur Isomerisierung von Harnstoff zu Cyanat auftrat.
4. Cyanat schützt einen Teil der gelösten Eiweisstoffe gegen irreversible Denaturierung durch Erhitzen oder Alkohol.

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5. Cyanat hat, wie festgestellt wurde, die Fähigkeit, denaturierte und gefällte Eiweissstoffe in Lösung zu bringen.

6. Die Erhöhung der Hitze koagulierungstemperatur von Eiweisskörpern, die von einer Anzahl Stoffen hervorgerufen wird, scheint mit der Fähigkeit dieser Stoffe, das Lösen denaturierten Eiweisses zu fördern, parallel zu laufen.

7. Wegen der wahrscheinlichen Cyanatbildung aus Harnstoff im Organismus wurde die Hitze koagulierungstemperatur von Sera mit erhöhtem Harnstoffgehalt untersucht.

8. Die mögliche physiologische Bedeutung der Resultate und ihre Beziehung zur antimittotischen Wirkung von Cyanat wird besprochen.

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## LA COMPOSITION DU LYSOZYME EN ACIDES AMINÉS

I. ACIDES AROMATIQUES,  
ACIDES DICARBOXYLIQUES ET BASES HEXONIQUES

par

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La possibilité d'obtenir du lysozyme cristallisé à l'état pur<sup>1, 2</sup> et d'en contrôler l'activité biologique<sup>3, 4</sup>, le caractère enzymatique de cette substance et son poids moléculaire relativement faible, rendent particulièrement intéressant son choix comme matériel pour l'étude de la structure d'une protéine.

L'étude de cette structure nécessite la connaissance préalable, aussi exacte que possible, de la teneur en Azote total, en Soufre et éventuellement en Phosphore, et de la composition en acides aminés de la protéine en question. Or, parce que les Auteurs qui se sont attachés à l'étude analytique du lysozyme n'ont eu jusqu'ici entre les mains qu'une substance beaucoup moins pure qu'ils ne pensaient, et utilisaient des procédés analytiques parfois défectueux, les rares indications concernant le lysozyme, que l'on trouve actuellement dans la bibliographie sont, comme on le verra plus loin, souvent en désaccord les unes avec les autres. Aussi nous attachons-nous, dans le présent travail, à donner une première série de résultats concernant la composition et la teneur en divers acides aminés du lysozyme pur. Nous avons étudié à ce point de vue plusieurs échantillons de lysozyme; les uns ont été préparés par nous-mêmes, comme il est dit ci-dessous; un autre provient de "Armour Laboratories", Chicago, Ill. D'après les indications fournies par ces laboratoires, cet échantillon a été préparé comme les nôtres, mais n'a été recristallisé que trois fois. Sa teneur en azote est donnée comme étant de 16.8%, et son activité, mesurée d'après GOLDWORTHY ET FLOREY<sup>5</sup> sur *Micrococcus lysodeikticus*, est de 1000000 d'unités/g. Nous distinguons par un astérisque les résultats obtenus à partir de cet échantillon.

Tous les résultats analytiques sont ici calculés pour du lysozyme sec (séchage à 56°, sous 0.1 mm Hg) et sans cendre. Dans le cas des chiffres trouvés dans la littérature, et cités à titre de comparaison, ceux-ci ont été recalculés, lorsqu'il a été nécessaire, de telle sorte qu'ils correspondent également à la protéine sèche et sans cendre.

## PRÉPARATION DU LYSOZYME

Le lysozyme est préparé à partir du blanc d'œuf selon la méthode de ALDERTON ET FEVOLD<sup>2</sup>. Le produit cristallisé obtenu directement au sein du blanc d'œuf par alcalinisation de ce dernier à pH 9.5, suivie de l'addition de 5 % de chlorure de sodium et maintenu à 4° pendant 4 à 5 jours, contient au plus 40 à 50 % de lysozyme. On recueille ce produit cristallisé par centrifugation, on le lave

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à deux reprises par une solution de chlorure de sodium à 5 % ajustée à pH 9.5 par addition de soude. On en extrait le lysozyme en dissolvant celui-ci par une solution diluée d'acide acétique, de pH 4.6 à 5.6. On centrifuge pour éliminer les parties non solubles qu'on lave deux fois à l'acide acétique dilué. On réunit les solutions acétiques dont le volume total doit être environ le huitième de celui du blanc d'œuf initial. On recristallise une première fois le lysozyme par addition de soude jusqu'à pH 9.5 et de 5 % de chlorure de sodium, et en maintenant 5 jours à 4°, comme pour la cristallisation initiale. On récolte les cristaux, puis, après les avoir lavés par la solution alcaline de chlorure de sodium on les redissout dans un volume d'acide acétique dilué égal environ au vingtième du blanc d'œuf initial. La solution obtenue n'est pas encore limpide; on la centrifuge, puis on la filtre, toujours en la maintenant à 4°. A la solution alors limpide, on ajoute 5 % de bicarbonate de sodium. Le lysozyme précipite, d'abord amorphe, puis il cristallise. On achève la purification par dissolutions dans l'acide acétique et précipitations par le bicarbonate, répétées encore quatre fois, en filtrant éventuellement les premières solutions acétiques. Les deux dernières doivent rester limpides. A partir de 150 œufs de taille moyenne, on obtient ainsi finalement environ 3 g d'un produit parfaitement cristallisé en prismes allongés<sup>2</sup> se dissolvant instantanément dans l'acide acétique dilué en donnant une solution parfaitement limpide. Son activité biologique, mesurée par son action lysante vis-à-vis de *Micrococcus lysodeikticus*, selon la technique de BOASSON<sup>3</sup>, et comparée à celle du blanc d'œuf dilué pris comme étalon, est égale à celle indiquée par ALDERTON *et col.*<sup>1</sup> pour le lysozyme électrophorétiquement pur. En outre, sa composition en acides aminés ne varie plus à partir de la quatrième recristallisation; cette remarque est particulièrement importante en ce qui concerne le tryptophane, beaucoup plus abondant que dans les autres protéines, et la méthionine qui manque au contraire complètement. Une contamination par une autre protéine, par l'ovalbumine, par exemple, dont les teneurs en tryptophane et en méthionine sont respectivement de 1.3 %<sup>6</sup> et de 5.0 %<sup>7</sup>, se manifesterait par une baisse apparente de la teneur en tryptophane et la présence apparente de méthionine dans le lysozyme.

#### DOSAGE DE L'AZOTE TOTAL

Le dosage de l'azote total du lysozyme exige soit que l'on tienne compte des cendres correspondant aux sels toujours plus ou moins entraînés au cours de la cristallisation, soit que l'on élimine auparavant totalement ces sels. L'élimination des sels se fait par dialyse contre l'eau distillée courante. Etant donné le poids moléculaire relativement faible du lysozyme, la dialyse provoque la perte d'une certaine quantité de la substance. On arrive néanmoins à obtenir une solution pratiquement débarrassée de sels, qui, desséchée sous vide à froid, fournit du lysozyme pur, sous forme d'une poudre blanche flocculente. Le dosage de l'azote total a été fait ici tout d'abord par micro-Kjeldahl, après 15 heures de minéralisation, en présence d'acétate mercurique comme catalyseur; on sait que la minéralisation des protéines au cours du micro-Kjeldahl reste parfois incomplète<sup>8</sup>; d'autre part, l'azote du tryptophane se montre particulièrement résistant à la minéralisation<sup>9</sup>. Or, le lysozyme contient, comme on le verra plus loin, des quantités élevées de tryptophane. Il nous a donc paru utile de confronter les résultats obtenus avec ceux fournis par le dosage de l'azote total par micro-Dumas. Les résultats obtenus correspondant à la moyenne de deux dosages, exprimés en azote total pour cent du produit sec et sans cendres, sont les suivants:

Micro-Kjeldahl: 16.6; 16.7\* ; Micro-Dumas: 16.7.

Nous considérons ainsi que la teneur en azote total exprimée en pour cent du lysozyme sec et sans cendres, est égale à 16.7. C'est cette valeur que nous utilisons dans le présent travail pour ramener au lysozyme pur les résultats analytiques. Cette valeur diffère nettement de celles publiées précédemment: 15.85<sup>10</sup>; 13.3<sup>11</sup>; 16.4<sup>12</sup>.

#### ANALYSE QUALITATIVE DES ACIDES AMINÉS DU LYSOZYME

Avant de procéder à l'analyse quantitative des acides aminés constituant le lysozyme, nous avons cherché à caractériser qualitativement leur présence. L'hydrolysat



chlorhydrique obtenu comme il est dit plus bas a été soumis à la chromatographie de partage bidimensionnelle sur papier, selon la méthode maintenant classique de CONSDEN, GORDON ET MARTIN<sup>13</sup>, en utilisant comme solvant, d'une part, du phénol, d'autre part, du butanol, tous deux additionnés de 0.1% de cupron. Les acides aminés suivants ont pu être mis nettement en évidence: glycolle, alanine, sérine, cystine, thréonine, valine, leucine, isoleucine, tyrosine, phénylalanine, acide aspartique, acide glutamique, lysine, arginine, histidine. Manquent: le tryptophane, détruit au cours de l'hydrolyse chlorhydrique, mais dont on verra qu'il existe en forte proportion dans la molécule de lysozyme, l'hydroxyproline qui semble ne pas exister, et la proline et la méthionine, qui n'existent certainement pas.

#### DOSAGE DU TRYPTOPHANE

A une quantité comprise entre 2 et 6 mg de lysozyme, on ajoute 2 ml d'une solution de soude N contenant 35 mg de gélatine, selon les indications de GRAHAM *et col.*<sup>14</sup>, l'ensemble étant placé dans un tube scellé que l'on maintient deux heures à 110° selon HESS ET SULLIVAN<sup>15, 16</sup>. Le dosage colorimétrique du tryptophane est fait à l'aide de p-diméthylaminobenzaldéhyde, par la méthode de BATES<sup>17</sup>. Il fournit les valeurs suivantes, exprimées en tryptophane pour cent de lysozyme:

7.5; 7.4; 7.5. Moyenne: 7.47.

Valeurs publiées précédemment: 2.4<sup>18</sup>; 8.0<sup>19</sup>.

Nous confirmons ici l'observation de GRAHAM *et col.*<sup>14</sup>, à savoir que lorsque du tryptophane pur est traité par la soude en tube scellé dans les conditions que nous venons d'indiquer, l'intensité et la stabilité de la coloration produite sont nettement accrues si le traitement a lieu en présence de gélatine; il ne s'agit pas là seulement d'une protection du tryptophane contre sa destruction: en effet, l'intensité de la coloration qui se forme à partir de tryptophane traité à chaud en présence de gélatine comme il vient d'être dit, est supérieure à celle que l'on obtient à partir du mélange des mêmes quantités de tryptophane et de gélatine non soumis à la chaleur (Tableau I).

TABLEAU I

INFLUENCE DU TRAITEMENT PAR LA CHALEUR DU MÉLANGE TRYPTOPHANE -|- GÉLATINE SUR LA COLORATION FOURNIE PAR LE TRYPTOPHANE DANS LA MÉTHODE DE BATES<sup>17</sup>

0.1 ml d'une solution aqueuse contenant 520  $\mu$ g de tryptophane sont ajoutés à 2 ml d'une solution de soude N contenant 2.5 % de gélatine.

A = Soumis à 110° en tube scellé pendant 2 heures.

B = Non traité.

D = Densité optique mesurée pour  $\lambda = 600 \text{ m}\mu$ , sous 1 cm.

Solution	D	
	I	II
A	0.768	0.787
B	0.690	0.714

Il est donc essentiel, comme le recommandent GRAHAM *et col.*<sup>14</sup>, de préparer un tube témoin constitué par du tryptophane pur et de la gélatine.

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D'autre part, Y. MOULÉ nous a aimablement communiqué les résultats de dosages de tryptophane exécutés selon la méthode de VOISENET<sup>20</sup> portant sur le lysozyme, provenant de Armour Laboratories, soit avant toute hydrolyse : 7.64 %\*, soit après hydrolyse alcaline : 7.83 %\*. Ces résultats, obtenus par une méthode analytique autre que celle que nous avons utilisée ici, confirment les nôtres d'une façon satisfaisante.

#### DOSAGE DE LA TYROSINE

Le dosage de la tyrosine est fait soit sur des hydrolysats alcalins, soit sur des hydrolysats acides du lysozyme.

*Dosage sur hydrolysats alcalins.* Une quantité voisine de 100 mg de lysozyme est traitée en tube scellé par 2 ml de soude 5 N pendant 18 heures à 110°. L'hydrolysats obtenu est ensuite soumis à l'analyse d'après FOLIN ET MARENZI<sup>21</sup>. Les résultats, exprimés en tyrosine pour cent de lysozyme sont les suivants :

3.85; 3.81; moyenne: 3.83.

Valeur publiée précédemment: 4.9<sup>18</sup>.

FRAENKEL-CONRAT *et col.*<sup>19</sup> ont indiqué une valeur chromogène maxima de 12.4 %, calculée en tryptophane, correspondant au dosage, par le réactif de FOLIN, d'après HERRIOT<sup>22</sup> de l'ensemble tyrosine + tryptophane sur le lysozyme hydrolysé par la pepsine, après dénaturation par la chaleur en milieu acide. Expriment en tryptophane la valeur de 3.83 ci-dessus, soit 4.32 et l'ajoutant à la valeur de 7.47 obtenue pour le tryptophane, on trouve pour la somme tyrosine + tryptophane, exprimée en tryptophane, 11.79 %; cette dernière valeur ne diffère que de 5 % de celle que FRAENKEL-CONRAT *et col.* ont mesurée directement. L'accord est satisfaisant entre ces deux déterminations faites par des voies différentes.

*Dosage sur hydrolysats acides.* Des dosages de tyrosine ont été exécutés sur la fraction "aromatique" des hydrolysats acides obtenus comme il est dit plus bas, par colorimétrie comme dans le cas des hydrolysats alcalins. Les résultats, exprimés en tyrosine pour cent de lysozyme, sont les suivants :

3.30; 3.17; 3.67. Moyenne: 3.38.

Ces résultats sont nettement inférieurs à ceux que l'on trouve après hydrolyse alcaline. Cette infériorité est l'indice d'une destruction sensible de la tyrosine. Si celle-ci paraît en effet résister à toute destruction par les acides à chaud en l'absence de tryptophane ou en présence de faibles quantités de cet acide aminé<sup>23</sup>, elle est au contraire entraînée vers une destruction partielle, de l'ordre de 10 à 20 %, quand elle se trouve, comme ici, en présence d'une quantité importante de tryptophane. Aussi est-ce seulement aux valeurs de tyrosine obtenues après hydrolyse alcaline que nous attribuons une signification précise.

#### DOSAGE DES BASES HEXONIQUES ET DES ACIDES DICARBOXYLIQUES

100 mg environ de lysozyme sont traités, en tube scellé, par 3 ml d'acide chlorhydrique 5.5 N à 110° pendant 24 heures. Le liquide obtenu est évaporé à sec, sous vide, à la température ordinaire, en présence d'acide sulfurique, d'anhydride phosphorique et de potasse en pastilles. Le résidu est repris par l'eau distillée, celle-ci est évaporée, cette opération étant répétée encore deux fois. Le résidu est repris par l'eau et légèrement alcalinisé (jusqu'à teinte nettement rose de la phénolphthaléine) par addition de lithine N (environ 1.5 ml). L'ammoniac déplacé est entraîné sous vide par la vapeur d'eau, recueilli et dosé. Les résultats exprimés en mg d'azote pour 100 mg de lysozyme sont les suivants :

1.69; 1.63; 1.71; 1.69. Moyenne: 1.68.

On sait que cet ammoniac correspond pour la majeure partie à l'azote des groupements amidés, et pour une part beaucoup plus faible, à la décomposition du tryptophane et de certains acides aminés tels que la sérine et la thréonine.

La solution débarrassée d'ammoniac est soumise à la série successive des chromatographies telles qu'elles sont décrites par FROMAGEOT, JUTISZ ET LEDERER<sup>24</sup>, dans l'ordre: adsorption sur silice, adsorption sur charbon et adsorption sur alumine. On obtient ainsi les fractions correspondant à chacun des éluats: bases hexoniques, acides aminés dicarboxyliques, acides aminés aromatiques, puis au filtrat final: acides aminés neutres autres que les dérivés aromatiques. Nous n'étudions ici que les acides aminés des trois premières fractions.

#### BASES HEXONIQUES

La chromatographie de partage sur papier d'une gouttelette prélevée sur la fraction élue de la silice, après concentration sous vide, montre que seules les bases hexoniques sont présentes dans cette fraction. Leur séparation est donc bien spécifique.

L'azote total de l'ensemble des bases hexoniques correspondant à 100 mg de lysozyme, est, en mg:

5.54; 5.61; 5.58. Moyenne: 5.58.

Le dosage de l'*histidine* se fait par la méthode de PAULI, selon la technique de MCPHERSON<sup>25</sup>. Etant donnée la faible proportion de l'*histidine* par rapport à l'*arginine* présente, il était nécessaire de vérifier que cette dernière ne gêne pas le dosage colorimétrique de l'*histidine*. Il en est bien ainsi. Les valeurs trouvées pour l'*histidine*, exprimées en base libre pour 100 de lysozyme, sont les suivantes:

0.97; 0.91; 1.02; 0.91. Moyenne: 0.95.

Il était indispensable de savoir si une destruction notable de l'*histidine* n'avait pas lieu au cours de l'hydrolyse. Nous avons donc traité par l'acide chlorhydrique dans les conditions décrites plus haut, un mélange des trois bases hexoniques et de tryptophane, dans des proportions voisines de celles où ces acides aminés existent dans le lysozyme. Après élimination et dosage de l'ammoniac formé, la solution a été soumise à une chromatographie sur silice, suivie d'une élution. L'*arginine* et l'*histidine* ont été dosées dans l'éluat. Les résultats obtenus sont indiqués dans le Tableau II.

TABLEAU II  
DESTRUCTION PARTIELLE DE L'HISTIDINE PAR CHAUFFAGE EN MILIEU ACIDE EN PRÉSENCE DE TRYPTOPHANE

Composition du mélange en acides aminés (mg)	Azote (mg)	Retrouvé par dosage	Perte %
Arginine base 12.00 . . . . .	3.86	12.20	0
Histidine base 0.89 . . . . .	0.24	0.77	13.5
Lysine base 4.31 . . . . .	0.82	—	—
Tryptophane 7.00 . . . . .	0.96	—	—

N de NH<sub>3</sub> formé: 0.087 mg.

N du filtrat: 0.973 mg.

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Il apparaît ainsi qu'une partie non négligeable de l'histidine est détruite au cours du traitement par l'acide chlorhydrique. Le chiffre de 0.95% obtenu pour la teneur du lysozyme en histidine doit donc être corrigé par addition de 0.13, ce qui donne, pour cette teneur, une valeur de 1.08. Il convient de remarquer que la correction en question ne peut être rigoureuse: l'histidine est en effet vraisemblablement plus labile quand elle est combinée dans la protéine que lorsqu'elle se trouve à l'état libre.

Valeur publiée précédemment: 2.6<sup>12</sup>.

Le dosage de l'arginine se fait par la méthode de SAKAGUCHI, selon la technique de MCPHERSON<sup>25</sup>. Nous avons vérifié que ce dosage n'est pas gêné par la petite quantité d'histidine présente. Les valeurs trouvées pour l'arginine exprimées en base libre pour cent de lysozyme, sont les suivantes:

12.8; 13.5; 13.5; 13.6. Moyenne: 13.3.

Valeur trouvée antérieurement: 11.6%<sup>12</sup>.

L'estimation de la lysine résulte du calcul suivant: N Lysine = N bases totales (5.58) — N Arginine (4.22) — N Histidine (0.31) = 1.05 mg.

D'où, pour la lysine, exprimée en pour cent de lysozyme: 5.5.

Valeur trouvée antérieurement: 5.8%<sup>12</sup>.

#### ACIDES AMINÉS AROMATIQUES

La chromatographie de partage sur papier d'une gouttelette prélevée sur la fraction élue du charbon, après concentration sous vide, montre que seules la tyrosine et la phénylalanine sont présentes, en tant qu'acides aminés, dans cette fraction. Il apparaît donc que la destruction du tryptophane est pratiquement totale dans les conditions d'hydrolyse réalisées ici, et que les substances qui en résultent ne réagissent pas à la ninhydrine.

L'azote total de la fraction élue du charbon, correspondant à 100 mg de lysozyme sec et sans cendres, est, en mg:

0.89; 0.80; 0.86. Moyenne: 0.85.

C'est sur cette fraction élue du charbon qu'est exécuté le dosage de la tyrosine, dont il a été question plus haut, et le dosage de la phénylalanine.

La faible intensité de la tache correspondant à la phénylalanine après chromatographie de partage sur le papier, indique que la proportion de cet acide aminé dans la fraction en question est inférieure ou au plus égale à celle de la tyrosine. Le dosage de la phénylalanine par colorimétrie selon la méthode de KAPPELLER-ADLER<sup>26</sup> ne peut se faire en présence de tyrosine; la destruction préalable de cette dernière par le permanganate de potassium, réalisable lorsque la quantité de tyrosine n'est pas trop importante par rapport à celle de la phénylalanine, n'aurait guère de sens ici, où la tyrosine est en excès par rapport à la phénylalanine. Nous avons donc déterminé la quantité de phénylalanine présente par un autre procédé: on verra plus loin que le nombre de résidus de tyrosine par molécule de lysozyme (poids moléculaire = 13900) est de 3. Le nombre de molécules de phénylalanine, d'après ce qui vient d'être dit, ne peut donc être égal qu'à un, deux ou au maximum trois. Nous avons donc fait des solutions de tyrosine et de phénylalanine correspondant, en ce qui concerne la tyrosine, à la concentration de la fraction élue du charbon provenant de l'hydrolyse du lysozyme, et contenant une,

deux ou trois molécules de phénylalanine pour trois molécules de tyrosine. La comparaison de l'intensité des taches fournies par ces solutions avec celle des taches fournies par la solution éluée du charbon, après chromatographie sur papier (chromatographie unidimensionnelle avec du butanol comme solvant) et traitement à la ninhydrine, nous a permis de décider sans hésitation possible que le lysozyme renferme deux molécules de phénylalanine pour trois molécules de tyrosine.

En ce qui concerne l'azote total de la fraction éluée du charbon, il convient de faire la remarque suivante: L'azote de la tyrosine (0.26 mg) + l'azote de la phénylalanine (0.20 mg) retranchés de l'azote total (0.85) de la fraction, laissent 0.39 mg d'azote correspondant aux produits de décomposition du tryptophane. Ce n'est que 38% de l'azote du tryptophane (1.02 mg). Des expériences, dans le détail desquelles nous n'entrerons pas ici, faites sur le comportement du tryptophane et de ses produits de décomposition au cours des différentes opérations dont il s'agit ici, montrent que la perte en azote du tryptophane est due, pour la plus grande part, à la rétention d'une fraction importante des produits de décomposition du tryptophane par le charbon. Une telle rétention a d'ailleurs été observée déjà par G. SCHRAMM ET J. PRIMOSIGH<sup>27</sup>. C'est à quelques irrégularités dans cette rétention que l'on doit surtout attribuer les variations observées dans l'azote total contenu dans les fractions éluées du charbon, fractions provenant de différentes opérations concernant cependant une même quantité initiale de lysozyme.

#### ACIDES DICARBOXYLIQUES

La chromatographie de partage sur papier d'une gouttelette prélevée sur la fraction éluée de l'alumine après concentration sous vide, montre que seuls en tant que substances réagissant à la ninhydrine l'acide aspartique et l'acide glutamique sont présents dans cette fraction. La séparation des acides dicarboxyliques est donc bien spécifique.

L'azote total de l'ensemble des acides dicarboxyliques correspondant à 100 mg de lysozyme est, en mg:

2.55; 2.67. Moyenne: 2.61.

Le dosage de l'*acide aspartique* est fait tout d'abord par la méthode de FROMAGEOT ET COLAS<sup>28</sup>, dérivant de celle indiquée autrefois par FROMAGEOT ET HEITZ<sup>29</sup>, rendue spécifique pour l'acide aspartique par la séparation préalable de cet acide des autres acides aminés générateurs d'acétaldéhyde. Les valeurs trouvées, pour cent de lysozyme, sont les suivantes:

10.2; 10.1. Moyenne: 10.2.

Une deuxième série de dosages a été exécutée par la méthode de FISHER, PARSONS ET MORRISON<sup>30</sup>, dans des conditions qui seront exposées en détail dans le travail suivant. La moyenne des résultats obtenus dans la mesure des surfaces de 19 taches correspond à une teneur en acide aspartique de 10.9% de lysozyme. Cette valeur est en excellent accord avec la précédente, obtenue par une voie tout à fait différente; la moyenne générale est ainsi de 10.6%. Aucune autre valeur concernant la teneur du lysozyme en acide aspartique ne semble avoir été publiée jusqu'ici.

Le dosage de l'*acide glutamique* est fait par la méthode de FISHER, PARSONS ET MORRISON<sup>30</sup>; les déterminations ont porté sur 9 taches. La moyenne des résultats obtenus correspond à une teneur en acide glutamique de 3.0% de lysozyme.

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Valeur trouvée antérieurement: 3.5%<sup>31</sup>.

En ce qui concerne l'azote total de la fraction élue de l'alumine, il convient de faire la remarque suivante: l'azote de l'acide aspartique (1.10 mg) + l'azote de l'acide glutamique (0.30 mg) retranché de l'azote total (2.61 mg) de la fraction, laisse 1.21 mg d'azote de nature non définie. Plusieurs observations nous ont montré que c'est seulement dans le cas du lysozyme, particulièrement riche en tryptophane, que l'on observe ainsi un excès de l'azote total de la fraction élue de l'alumine, sur l'azote correspondant à la somme des deux acides dicarboxyliques. Nous en concluons que cet azote en excès correspond à des produits de décomposition du tryptophane.

#### DISCUSSION DES RÉSULTATS

L'ensemble des résultats précédents est groupé dans le Tableau III.

TABLEAU III

TENEUR DU LYSOZYME EN DIVERS ACIDES AMINÉS

Le teneur du lysozyme en azote total est considérée comme égale à 16.7%.

Acide aminé	Acide aminé pour 100 de lysozyme		Poids moléculaire minimum du lysozyme	Nombre de Résidus	Poids moléculaire du lysozyme
	Calculé	Trouvé			
Tryptophane . . .	7.33	7.47	2 730	5	13 650
Tyrosine . . . . .	3.91	3.83	4 720	3	14 150
Phénylalanine . .	2.37	—	—	2	—
Ac. aspartique . .	10.5	10.6	1 380	11	13 800
Ac. glutamique . .	3.17	3.0	—	3	—
Arginine . . . . .	13.7	13.4	1 300	11	14 300
Histidine . . . . .	1.11	1.08	14 350	1	14 350
Lysine . . . . .	5.25	5.5	2 650	5	13 250
Moyenne:			.		13 900

Ces premiers résultats permettent en premier lieu de préciser le poids moléculaire du lysozyme. Ce poids moléculaire a été donné comme étant successivement de 25 000<sup>10</sup>, 18 000<sup>12</sup>, 17 500<sup>1</sup>, 14 000 à 17 000<sup>1</sup>, 13 000<sup>32</sup> et 13 900  $\pm$  600<sup>33</sup>.

A priori, la valeur la plus sûre doit être 13 900  $\pm$  600 qui est fournie par une étude de la diffraction des rayons X. Les résultats analytiques obtenus ici sont en excellent accord avec cette dernière valeur. Le dosage de l'histidine et celui de la tyrosine, dont il existe respectivement un et trois résidus par molécule de lysozyme, sont particulièrement intéressants à ce point de vue.

En ce qui concerne la valeur trouvée pour l'acide glutamique, celle-ci est sensiblement inférieure à 3.17%, valeur qui correspondrait à la présence de trois résidus d'acide glutamique dans une molécule de lysozyme de poids moléculaire de 13 900. Il semble néanmoins que le nombre de résidus d'acide glutamique soit bien ici de trois; le fait de trouver après hydrolyse acide une valeur inférieure, peut s'expliquer en effet par la destruction de l'acide glutamique au cours de cette hydrolyse, notamment par sa cyclisation bien connue en acide pyrrolidone-carbonique. Le dosage de l'acide glutamique a été effectué ici par comparaison avec les données fournies par un mélange d'acide glutamique et de tryptophane, en proportion convenable, traité par l'acide chlor-

hydrique dans les conditions de l'hydrolyse; mais il est vraisemblable que la labilité de l'acide glutamique combiné dans le lysozyme est supérieure à celle du même acide à l'état libre.

D'autre part, en ce qui concerne la teneur en acides dicarboxyliques totaux, celle-ci correspond à la présence de 14 groupements carboxyliques en  $\omega$ . Or, d'après les données de FRAENKEL-CONRAT<sup>34</sup>, on peut calculer que le lysozyme contient 14 groupements amides pour un poids moléculaire de 13900. La molécule de lysozyme ne renfermerait ainsi aucun groupement carboxylique libre, les acides aspartique et glutamique s'y trouvant entièrement sous forme de leurs amides respectifs.

D'autre part enfin, le lysozyme apparaît comme une protéine particulièrement riche en tryptophane. Si l'on ne tient pas compte de la gramicidine, qui est un polypeptide et non une protéine, on ne connaît guère, jusqu'ici, que le venin d'abeille qui présente une teneur comparable en tryptophane<sup>35</sup>.

### RÉSUMÉ

L'analyse du lysozyme pur, cristallisé, préparé à partir du blanc d'œuf selon la méthode de ALDERTON ET FEVOLD, a été faite en utilisant la technique de séparation des acides aminés en groupe, qui a été décrite précédemment par FROMAGEOT, JUTISZ ET LEDERER. Cette analyse porte ici sur les acides aminés aromatiques, les bases hexoniques et les acides dicarboxyliques. Les résultats obtenus, exprimés en résidus par molécule de lysozyme de poids moléculaire égal à 13900, sont les suivants: tryptophane 5, tyrosine 3, phénylalanine 2, acide aspartique 11, acide glutamique 3, arginine 11, histidine 1, lysine 5. Les valeurs trouvées, en particulier pour l'histidine et la tyrosine, permettent de déterminer un poids moléculaire (13900) en excellent accord avec celui que PALMER, BALLANTYNE ET GALVIN ont calculé à partir des données fournies par la diffraction des rayons X.

### SUMMARY

Pure crystallized lysozyme, prepared from egg white using the method of ALDERTON AND FEVOLD, has been analysed by separation of the amino-acids into groups, as described by FROMAGEOT, JUTISZ, AND LEDERER. The analysis concerned aromatic amino-acids, hexonic bases and dicarboxylic acids. The results expressed in radicals per molecule of lysozyme are as follows: tryptophan 5, tyrosine 3, phenylalanine 2, aspartic acid 11, glutamic acid 3, arginine 11, histidine 1, lysine 5. These figures, especially the ones for histidine and tyrosine, allow the determination of a molecular weight (13900) in excellent agreement with that calculated by PALMER, BALLANTYNE, AND GALVIN from the results of X-ray diffraction spectra.

### ZUSAMMENFASSUNG

Reines, kristallisiertes Lysozym, welches aus Eiweiss nach der Methode von ALDERTON UND FEVOLD hergestellt worden war, wurde nach der von FROMAGEOT, JUTISZ UND LEDERER früher beschriebenen Trennungsmethode der Aminosäuren in Gruppen analysiert. Diese Analyse erfasst hier die aromatischen Aminosäuren, die Hexonbasen und die zweibasischen Säuren.

Die erhaltenen Resultate sind in Resten pro Lysozymmolekül vom Molekulargewicht 13900 wie folgt ausgedrückt: Tryptophan 5, Thyrosin 3, Phenylalanin 2, Asparaginsäure 11, Glutaminsäure 3, Arginin 11, Histidin 1, Lysin 5.

Aus diesen Werten, insbesondere aus denjenigen für Histidin und Thyrosin, kann für das Molekulargewicht ein Wert (13900) bestimmt werden, der mit dem von PALMER, BALLANTYNE UND GALVIN aus Röntgenstrahlendiffraktionen errechneten ausgezeichnet übereinstimmt.

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## CLEAVAGE RATE, OXYGEN CONSUMPTION AND RIBOSE NUCLEIC ACID CONTENT OF SEA URCHIN EGGS

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### I. INTRODUCTION

At a given temperature the cleavage rates of the eggs of various sea urchin species may differ widely from one another. The nature of the determination of these differences in cleavage rate is not only of general interest but has also practical importance when it is recalled that genetically determined growth rate differences within a single species are reflected in the cleavage rates of the embryonic cells (c.f. PAINTER<sup>1</sup>).

A number of investigators have studied the cleavage rate of sea urchins' eggs, this material offering many special facilities for experimental attack. The early work of DELAGE, GODLEWSKI, NEWMAN, TENNENT and others, which gave results conflicting in many particulars, has been reviewed by MOORE<sup>2</sup>, and the reader is referred to his account.

One line of experiment was the study, within a species, of the relative contributions made by egg cytoplasm, egg nucleus and sperm nucleus to the determination of cleavage rate.

DELAGE<sup>3</sup> cut virgin eggs of *Strongylocentrotus lividus* into nucleated and non-nucleated fragments: when such fragments were subsequently fertilized, the diploid fragment was found to cleave somewhat more slowly than whole egg controls, and the haploid fragment still more slowly.

WHITAKER<sup>4</sup>, working with eggs of the starfish *Patiria miniata*, repeated DELAGE's experiment and confirmed his results. He suggested, however, that the effect might be due to inequalities of yolk distribution in nucleated and non-nucleated fragments rather than directly associated with the nuclear differences. TENNENT, TAYLOR, AND WHITAKER<sup>5</sup> also repeated DELAGE's experiments. In this case the material consisted of eggs of *Lytechinus variegatus*, and once more DELAGE's results were confirmed.

WHITAKER<sup>7</sup> went on to study the rate of cleavage of egg fragments of *Arbacia* which, prior to fragmentation, had been stratified by centrifugation. The cuts were made so as to sever the "light" end of the egg, with nucleus, from the "heavy" anucleate end. The differential distribution of various cytoplasmic materials was found to make no difference to cleavage rate, the centrifuged fragments cleaving at exactly the same rates as comparable non-centrifuged fragments. However, the delay of haploid over diploid fragments was once more confirmed (in this case diploid fragments were found to cleave slightly faster than whole egg controls), and WHITAKER concluded that the nuclear/cytoplasmic ratio is a determining factor in cleavage rate. However, the differ-

ences of rate observed by WHITAKER were in fact very small indeed (of the order of 6 min in 50), and later evidence, which we shall now consider, indicates that his conclusions were not justified.

MOORE<sup>2</sup> has made a very elegant experimental analysis which has exposed the roots of the problem. Advantage was taken of the fact, already mentioned, that the eggs of different species of sea urchins cleave at different rates. The eggs of *Strongylocentrotus franciscanus* at 20° C take approximately 95 min to pass from fertilization to the first cleavage, and succeeding divisions take about 47 min each. The eggs of *Dendraster excentricus* divide nearly twice as rapidly, the first cleavage occurring after 57 min, and the interval between subsequent divisions being approximately 28 min. When MOORE crossfertilized these two species he found that the cleavage rate of the hybrids was precisely that characteristic of the egg and independent of the sperm. This experiment fails to differentiate between the contribution of the egg cytoplasm and egg nucleus. To answer this question, MOORE cut unfertilized eggs into pieces and then made cross-fertilizations. All such fragments, with or without the egg nucleus, were found to cleave at the rate characteristic of the original eggs. These experiments show not only that it is the egg cytoplasm which determines cleavage rate, but also that this rate is, at least within certain limits, independent of egg size. MOORE interprets WHITAKER's results as being ascribable to slight inequalities in the distribution of some critical cytoplasmic material between haploid and diploid egg fragments.

The question which next presents itself is whether by means of experiment, a factor determining cleavage rate can actually be located within the egg cytoplasm. Such a substance had already been envisaged by LOEB AND CHAMBERLAIN<sup>6</sup> in their attempt to provide a physico-chemical explanation for inter-egg variability of cleavage rate within a single species. The centrifuge experiments of E. B. HARVEY<sup>8, 9</sup> provide relevant information. Under the action of centrifugal force *Arbacia punctulata* eggs stratify into five layers: oil, clear layer, granular layer (the granules being identified as mitochondria), yolk layer and pigment. The oil cap is at the centripetal, the pigment at the centrifugal end of the egg, while the nucleus lies in the clear layer. With stronger centrifuging the stratified eggs may be pulled apart into "lighter" and "heavier" half-eggs, the lighter half containing oil, nucleus, clear layer and part of the granular layer, the heavier half containing the rest of the granular layer together with yolk and pigment.

The light half-eggs, when fertilized, cleave at approximately the same rate as whole eggs, though occasionally the rate is perceptibly higher. The heavy half-eggs, when fertilized, undergo cycles of nuclear division which are somewhat delayed as compared with whole eggs. These nuclear divisions are not at first accompanied by cytoplasmic cleavage; however later the cytoplasm splits up between the nuclei. HARVEY recentrifuged the light half-eggs, which then separate into quarter-eggs, the centripetal quarter-egg containing oil, nucleus and part of the clear layer, while the centrifugal quarter-egg contains the rest of the clear plus the granular layer. When these quarter-eggs are fertilized the nucleated quarter cleaves exceedingly slowly: the granular quarter, on the other hand, cleaves at nearly the same rate as whole eggs. HARVEY<sup>9</sup> found the same rules to hold for centrifuged eggs of *Arbacia lixula* and *Sphaerechinus granularis*. The situation is, however, somewhat different in two other species *Paracentrotus lividus* and *Psammechinus microtuberculatus*, which do not stratify in the same order as do *Arbacia* and *Sphaerechinus*.

MOORE<sup>10</sup> applied HARVEY's centrifuge technique to the eggs of *Dendraster excent-*

*tricus*. The eggs were stratified and pulled into dumb-bell shape but not completely split into fragments. They were then activated parthenogenetically. In these stratified eggs the anucleate centrifugal end always cleaved in advance of the nucleate centripetal end, while in some egg batches so treated, no cleavage of the centripetal end took place at all. Topographically speaking, MOORE's results are the antithesis of HARVEY's results with *Arbacia* and *Sphaerechinus*, but this may well be due to differences in the order of stratification of the various cytoplasmic components. On the basis of HARVEY's results and his own, MOORE concludes that under the action of centrifugal force a "cleavage-substance" can be differentially distributed through the egg cytoplasm and that the rates of cleavage of the various egg regions or fragments are then determined by the relative concentrations of this substance.

However, one point which emerges from the work of HARVEY AND MOORE has been, in my opinion, somewhat overlooked by the latter author. If the word "cleavage-substance" is used to define some stratifiable material of the egg cytoplasm whose concentration is the limiting factor determining normal cleavage rate, then it should be possible not only to decrease its concentration, thereby decreasing the rate, but also to increase its concentration, in which case a faster rate should result. Cleavage rate can undoubtedly be reduced by stratification: but at the same time there is no experimental evidence for a clear cut and striking increase in cleavage rate in any way comparable to the interspecific differences which exist in nature. In the absence of this evidence the reduction of cleavage rate observed in egg fragments and regions produced by centrifugation is better envisaged as the imposition of a new limiting factor rather than the differential distribution of a factor limiting the normal cleavage rate in the entire egg. I therefore suggest that the use of the word "cleavage substance" be discontinued or at least reserved until such time as a normal limiting factor is discovered.

There remains another line of work which bears directly on cleavage rate determination and which we must consider before turning to the experimental results reported in this paper. HÖRSTADIUS<sup>11</sup> has shown that the winter and summer eggs of *Paracentrotus lividus* cleave at different rates when exposed to the same temperature. At 13° C the winter eggs cleave faster than the summer eggs. At 26° C the summer eggs cleave faster than the winter eggs. This phenomenon, which may be best described as acclimatization to the normal environmental temperature, has also been studied by FOX<sup>12, 13</sup> from a somewhat different point of view. FOX determined the cleavage rates of closely related species of *Psammechinus* and the same species of *Paracentrotus* from different localities. He found that at a given temperature (ca. 20° C) the material deriving from colder waters cleaved more rapidly than that deriving from warmer waters.

It will thus be clear that although there are specific differences in cleavage rate, these rates are not rigidly controlled and may undergo adaptation. HÖRSTADIUS has suggested that this adaptation involves alterations in the dispersion of the protoplasmic colloids.

## II. CLEAVAGE RATES OF THE NEAPOLITAN SEA URCHIN SPECIES

*Psammechinus microtuberculatus* (Blainv.), *Sphaerechinus granularis* (Lam.), *Arbacia lixula* (L.) and *Paracentrotus lividus* (Lam.) are four species of sea urchins which occur commonly in the Gulf of Naples. For the purposes of the experiments to be described in this paper, these species were used during the months of March and April 1947.

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TABLE I

Species	Individual	Time in minutes between:		
		fertilization and first cleavage	first and second cleavage	second and third cleavage
<i>Psammechinus microtuberculatus</i> (Blainv.)	A	58	38	34
		58	37	35
	B	63	37	33
		62	37	34
	C	63	37	36
		62	39	37
	Averages	61.0	37.5	34.8
<i>Paracentrotus lividus</i> . . . . . (Lam.)	A	74	43	43
		74	43	44
	B	79	43	45
		79	43	45
	C	76	42	45
		77	43	—
	D	74	43	44
		75	42	44
	Averages	76.0	42.7	44.3
<i>Arbacia lixula</i> . . . . . (L.)	A	97	54	52
		100	53	55
	B	95	52	57
		97	52	58
	C	102	53	55
		105	57	54
	D	99	56	55
		100	56	55
	Averages	99.4	54.1	55.1
<i>Sphaerechinus granularis</i> . . . . . (Lam.)	A	103	57	56
		101	57	58
	B	99	59	54
		100	58	52
	C	99	58	57
		101	57	55
	Averages	100.5	57.7	55.3

Eggs and sperm were collected by clipping away the oral half of adult specimens and inverting the aboral half over bowls containing filtered sea water. This method of egg collection is preferable to the excision of whole ovaries, as the egg batches so obtained contain hardly any immature oocytes.

The egg batches were repeatedly rinsed with large quantities of clean sea water, and trial inseminations were made about one hour after shedding. Occasionally, for reasons which are not apparent, seemingly mature eggs cannot be fertilized. Such egg batches were rejected. When trials showed that effectively 100% fertilization followed insemination, small quantities of eggs from the same parent batch were transferred to 100 ml Erlenmeyer flasks containing about 40–50 ml of clean sea water: the flasks were then placed in a water bath maintaining a temperature of  $18^{\circ} \pm 0.2^{\circ} \text{C}$ . The temperature of the circulating sea water of the laboratory was at this period about  $13^{\circ} \text{C}$ . After equilibrating for half an hour, small quantities of sperm were pipetted into the flasks, and the contents momentarily agitated to ensure even distribution of sperm and eggs.

The rate of development of the fertilized eggs was followed at regular interval by pipetting out a small sample and examining it under the microscope. In the conditions of the experiment outlined above, the rate of development is remarkably regular; no attempt was therefore made to treat the material statistically. The time of the first cleavage was taken as being that where 50% of the eggs in any one sample showed a well-marked cytoplasmic furrow, the remaining 50% being uncleaved. A similar criterion was used for the second and third cleavage stages. In Table I the time intervals between fertilization and the first, second and third cleavages are tabulated to the nearest minute. Any one entry would require the figures  $\pm 2$  to 3 min in order to include practically all of the slowest and fastest cleaving eggs from the batch in question. The figures show that *Psammechinus* is the fastest cleaving species, *Arbacia* and *Sphaerechinus* cleave most slowly and at about the same rate as one another, while *Paracentrotus* falls between the two extremes.

In a short series of subsidiary experiments, *Psammechinus* at  $13^{\circ} \text{C}$  was found to cleave at the same rate as *Arbacia* and *Sphaerechinus* at  $18^{\circ} \text{C}$ . However, the converse experiment was not possible:  $28^{\circ} \text{C}$  proved to be the upper limit of temperature for *Sphaerechinus* egg development, and at no temperature below this limit does *Sphaerechinus* cleave as rapidly as does *Psammechinus* at  $18^{\circ} \text{C}$ .

### III. OXYGEN CONSUMPTION DURING CLEAVAGE

GRAY<sup>14</sup> has stated that the rate of cell division during segmentation of the egg of *Echinus miliaris* bears no obvious relationship to the rate of metabolism during this process. Thus, though the early cleavage divisions occur after equal intervals of time, the cleavage rate being therefore constant, the rate of oxygen consumption slowly rises as more and more reserve material is incorporated into the respiring protoplasm.

However, despite the absence of a direct correlation between cleavage and metabolic rate, we are bound to admit that the two processes are very intimately related to some common denominator. This conclusion derives from the experiments of EPHRUSSI<sup>15</sup> and TYLER<sup>17</sup> who studied the oxygen consumption and developmental rates of various echinoderm eggs at different temperatures. These authors found that the total quantity of oxygen consumed in reaching a given developmental stage is the same at different temperatures. In other words, the temperature coefficients of cleavage and respiration

are identical, and the two processes cannot be dissociated by varying the culture temperature, provided that this remains within the physiological range.

The following experiment was designed in order to determine whether the relationship between cleavage and metabolic rate within a species established by EPHRUSSI AND TYLER can be extended to cover related species whose cleavage rates differ.

Eggs from a number of females of one of the four sea urchin species were collected. Samples were tested for maturity by trial inseminations, and those eggs batches which passed the test were pooled together, washed in filtered sea water and fertilized in bulk. The fertilized eggs were then thoroughly washed four or five times with fresh sea water and finally concentrated in about 40 ml volume. The concentration was checked roughly by centrifuging a small sample.

The final egg suspension was thoroughly mixed and pipetted, 3 ml at a time, into five WARBURG respirometer vessels. Aliquot volumes of the same suspension were pipetted into three centrifuge tubes and three KJELDAHL flasks.

The central chambers of the WARBURG respirometer vessels were fitted with filter-paper rolls wetted with 6 drops of 5 N potassium hydroxide, and the vessels attached to their respective manometers. They were then placed on rocking racks in a thermostatically-controlled water bath at 18° C and rocked gently during the course of the observations. The first manometer readings were taken two hours after the time of fertilization and continued at ten minute intervals for a further two and a half hours. At the end of each run the eggs were checked for regularity of development. Out of over twenty separate determinations of oxygen consumption, two were rejected on the score of developmental irregularities.

The manometric readings were plotted graphically, after conversion to volumes at N.T.P., as total oxygen consumption against time. The curves so obtained (cf. GRAY<sup>17</sup>) are effectively linear over the time range 2-4 ½ hours after fertilization and were treated as such.

Aliquot volumes of egg suspension pipetted into centrifuge tubes were to serve for dry weight determinations. Since the eggs of sea urchins are surrounded by a mucilaginous jelly-coat which otherwise makes a considerable "dead weight" contribution to dry weight, the jelly-coats were removed by suspending the eggs for fifteen minutes in 0.52 M sodium chloride solution. This treatment, coupled with mechanical agitation, is generally adequate. The stages in the removal of the jelly-coat can be readily observed under the microscope if a small quantity of Indian ink be added to the fluid in which the eggs are lying. I am indebted to Dr. MONROY, of the Stazione Zoologica, for details of this useful technique (cf. MONROY AND RUFFO<sup>18</sup>).

The eggs, freed from their jelly-coats, were again suspended in sea water, and then strongly centrifuged. The supernatant water was removed at the pump and all but the last traces absorbed at the sides of the centrifuge tubes by filter paper. The eggs and tubes were then dried to a constant weight in an oven at 110° C. Owing to the impossibility of removing all sea water before drying the material, the dry weight determinations are slight overestimates of the true dry weight of eggs. However, the salt error involved has been calculated to represent less than 5% of these determinations.

The aliquot volumes of egg suspension in KJELDAHL flasks were also treated so as to remove the jelly-coats from the eggs. Total nitrogen determinations were subsequently made by a micro-KJELDAHL method.

The results of this experiment are given in detail in Table II. It will be seen that

TABLE II

Species	Oxygen consumption per hour ( $\mu$ l)	Aliquot dry weight determinations (mg)	Oxygen consumption per hour per 100 mg dry weight ( $\mu$ l)	Aliquot total nitrogen determinations (mg)	Oxygen consumption per hour per 1 mg total nitrogen ( $\mu$ l)
<i>Psammechinus microtuberculatus</i> (Blainv.)	2.50	44		2.59	
	2.66	46		2.95	
	2.54	50		2.98	
	2.79	52			
	2.37				
Averages	2.57	48	5.35	2.84	0.90
<i>Paracentrotus lividus</i> (Lam.)	3.13	66		4.58	
	3.18	71		4.88	
	3.51	75			
	3.45				
	3.16				
Averages	3.29	71	4.63	4.73	0.70
<i>Arbacia lixula</i> (L.)	3.55	94		6.13	
	3.34	94		6.55	
	3.80	96		6.61	
	3.37	96			
Averages	3.51	95	3.70	6.43	0.55
<i>Sphaerechinus granularis</i> (Lam.)	4.11	94		6.40	
	4.69	101		6.52	
	4.49	102			
	4.43				
Averages	4.43	99	4.47	6.46	0.69

TABLE III

Species	Average time interval between successive cleavages (min)	Oxygen consumption per hour per 100 mg dry weight ( $\mu$ l)	Oxygen consumption between successive cleavages per 100 mg dry weight ( $\mu$ l)
<i>Psammechinus</i> (18° C)	36.1	5.35	3.22
<i>Psammechinus</i> (13° C)	60.0	3.18	3.18
<i>Paracentrotus</i> (18° C)	43.2	4.63	3.34
<i>Arbacia</i> (18° C)	54.6	3.70	3.37
<i>Sphaerechinus</i> (18° C)	56.5	4.45	4.19

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*Psammechinus* respire most rapidly of the four species, *Arbacia* most slowly, while *Paracentrotus* and *Sphaerechinus* lie between the two extremes and have equal rates of respiration.

In Table III the cleavage and respiration rates of the four species are set beside one another together with the computed quantities of oxygen consumed between two successive cleavages. The data from a single respiration determination for *Psammechinus* at 13° C is also included. (At this temperature *Psammechinus* cleaves at the same rate as do *Arbacia* and *Sphaerechinus* at 18° C). While *Psammechinus*, *Paracentrotus* and *Arbacia* absorb comparable amounts of oxygen between successive cleavages per unit of dry weight, *Sphaerechinus* is out of line in having a considerably higher oxygen requirement.

The exceptional behaviour of *Sphaerechinus* bears out GRAY's contention that there is no direct and simple correlation between metabolic and cleavage rates. It may be that this species possesses proportionately less non-respiring reserve materials. Nevertheless, the identity in oxygen requirement of the other three species when performing a comparable act of development should not be overlooked since it may indicate that, other things being equal, the metabolic cost of cleavage is proportional to its rate.

#### IV. THE RIBOSE NUCLEIC ACID CONTENT OF SEA URCHIN EGGS

On the basis of HARVEY's experiments with half- and quarter-eggs produced by centrifugal force, MOORE has argued that the mitochondrial granules may represent the "segmentation stuff" of LOEB AND CHAMBERLAIN, whose concentration in the cytoplasm determines cleavage rate. In the introduction to this present paper the logic of MOORE's argument has been questioned: however, since ribose nucleic acid has been shown by BRACHET and by CASPERSSON to be a characteristic component of rapidly dividing tissues, (see BRACHET<sup>19</sup>) and since CLAUDE<sup>20</sup> has put forward the view that the granules rich in ribose nucleic acid are, in fact, the mitochondria of cytologists, I thought it of some interest to determine the relative concentrations of ribose nucleic acid in the virgin eggs of the four neapolitan sea urchin species.

The determinations were made according to the method of BRACHET<sup>22</sup>. Mature virgin eggs were obtained from a number of females. The eggs were thoroughly washed in clean sea-water, and the jelly-coats removed by the method already described. The eggs were then washed once more, concentrated into a small volume of sea water and fixed in a 10% solution of trichloroacetic acid. After prolonged extraction in three changes of this solution the eggs were pipetted on to weighed fragments of coverslips and dried out to constant weight at 110° C. Neutral fats were then removed by extraction in hot ether, and the resultant material dried and weighed.

The weighed samples were then hydrolysed and steam distilled in a sulphuric acid-zinc sulphate-potassium sulphate mixture, as described by BRACHET: the furfural content of the resulting distillate was subsequently determined by means of a PULFRICH photometer, use being made of the coloured product formed between furfural, aniline and acetic acid. Among the various sources of error involved in this technique, the following are noteworthy:

a. Jelly-coats must be completely removed before fixation of the eggs, not only because they contribute to the figures of dry weight, but also because they generate furfural on hydrolysis.



b. Prolonged extraction in trichloro-acetic acid is necessary to remove soluble sugars and glycogen which would otherwise also yield furfural.

c. Failure to extract neutral fat leads to turbidity in the distillate and hence to inaccuracies in the colorimeter readings.

The results of the determinations are given in detail in Table IV. (For the calculation of ribose nucleic acid content, 1303 parts by weight of this substance are taken as yielding 384 parts by weight of furfural).

From these figures it is evident that the content of ribose nucleic acid cannot be the sole or even the overriding determinant of the cleavage interval. This does not exclude the possibility that it plays a role in the whole physiological system on which cleavage depends, but for the solution of this question, interspecific comparisons are not an adequate method.

TABLE IV

Species	Dry weight of ether extracted virgin eggs (mg)	Yield of furfural ( $\gamma$ )	Estimated ribose nucleic acid (mg per g)
<i>Psammechinus microtuberculatus</i> (Blainv.)	51.4	19.6	1.30
	41.5	19.2	1.57
	50.1	21.2	1.44
	39.1	16.6	1.44
	37.2	15.1	1.37
			Average 1.42
<i>Paracentrotus lividus</i> (Lam.)	39.6	11.0	0.94
	39.1	9.0	0.78
	38.4	8.5	0.76
	39.7	11.1	0.95
	37.7	9.6	0.86
			Average 0.86
<i>Arbacia lixula</i> (L.)	39.4	11.2	0.97
	40.4	12.4	1.04
	36.5	10.9	1.01
	41.8	12.1	0.98
			Average 1.00
<i>Sphaerechinus granularis</i> (Lam.)	41.0	16.4	1.36
	39.8	14.0	1.20
	51.9	21.2	1.39
	32.6	13.1	1.37
	26.8	10.9	1.38
	27.8	11.8	1.44
			Average 1.36

#### V. THE ORDER OF STRATIFICATION OF THE CYTOPLASMIC COMPONENTS

In the preceding parts of this paper it has been shown that differences in cleavage rate of sea urchin eggs cannot be directly related either to differences in total

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metabolic rate or to differences in the initial ribose nucleic acid content prior to cleavage.

There remains, however, another property of these sea urchin eggs which differs in a graded way between the species, and the seriation is in accordance with the seriation of the cleavage rates.

HARVEY<sup>9</sup>, by means of the centrifuge technique, was able to show that the cytoplasmic components of the eggs stratify in different order in different species. I have repeated her experiment and have obtained similar results. The order of stratification is shown diagrammatically in Fig. 1. Working from the centripetal to the centrifugal ends of the eggs, the fast-cleaving *Psammechinus* egg is stratified into oil, yolk (with nucleus), clear zone and mitochondria; the *Paracentrotus* egg into oil, clear zone (with nucleus), yolk and mitochondria; the slow cleaving *Arbacia* and *Sphaerechinus* eggs into

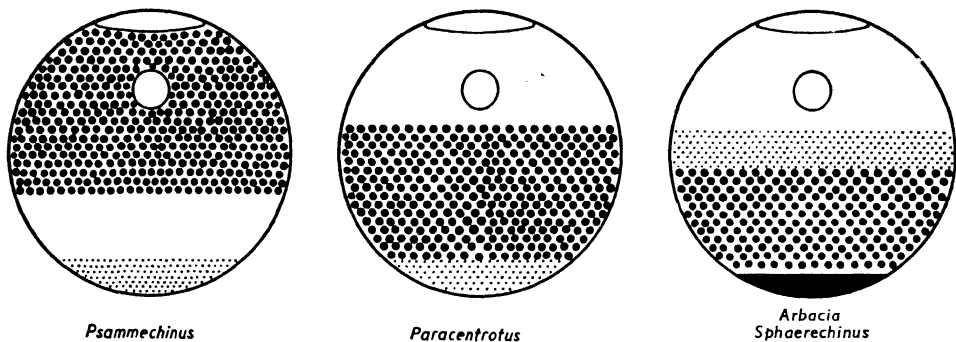


Fig. 1. Diagrammatic representation of the stratified cytoplasmic components of sea urchins' eggs: centripetal pole, with oil cap, is uppermost; coarse granules are yolk, fine granules mitochondria; the black area represents pigment in *Arbacia* and "heavy" clear cytoplasm in *Sphaerechinus*.

oil, clear zone (with nucleus), mitochondria and yolk. Thus the relative density of the yolk granules is least in *Psammechinus*, greatest in *Arbacia* and *Sphaerechinus* with *Paracentrotus* occupying the intermediate position.

It would be entirely premature to discuss this possible correlation, which may be purely fortuitous, before appropriate experiments have been undertaken to test its validity. A direct correlation between relative yolk density and cleavage rate is improbable, considering what is known of the cleavage rates of non-yolky half-and quarter-eggs. However the correlation may well be an indirect one, depending on the density or some linked property of another of the stratifiable cytoplasmic components.

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#### SUMMARY

1. There are striking differences in the cleavage rates of the eggs of four neapolitan sea urchin species.

2. These cleavage rates are not directly correlated with differences in the rate of oxygen consumption of the eggs during the early cleavage stages, nor

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3. are they correlated with differing ribose nucleic acid concentrations in the virgin eggs.
4. There is a hint that cleavage rate differences may be correlated with variations in the order of stratification of the cytoplasmic constituents when eggs are subjected to centrifugal force.

## RÉSUMÉ

1. Il existe des différences frappantes dans les vitesses de division des œufs de quatre espèces napolitaines d'oursins.
2. Ces différences ne correspondent ni à des différences dans les intensités de consommation d'oxygène au cours des premiers stades de division,
3. ni à des différences dans les concentrations des œufs vierges en acide ribonucléique.
4. L'action de la force centrifuge semble montrer que ces différences de vitesse de division dépendent de variations dans la stratification des constituants cytoplasmiques.

## ZUSAMMENFASSUNG

1. In den Spaltungsgeschwindigkeiten der Eier von vier napolitanischen Seeigelarten treten starke Unterschiede auf.
2. Diese Spaltungsgeschwindigkeiten hängen nicht direkt mit Unterschieden in der Rate des Sauerstoffverbrauchs in den frühen Spaltstadien zusammen, noch
3. sind sie von verschiedenen Ribosenukleasekonzentrationen in den unbefruchteten Eiern abhängig.
4. Es sind Anzeichen vorhanden, dass die Spaltungsgeschwindigkeitsunterschiede von Variationen in der Streckungsordnung der Zytoplasmabestandteile, wenn die Eier der Zentrifugalkraft unterworfen werden, abhängig sein könnten.

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## SOME PHYSICO-CHEMICAL PROPERTIES OF THYMO-NUCLEOPROTEIN PREPARED ACCORDING TO MIRSKY AND POLLISTER

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The first methods for the preparation of nucleoproteins were rather drastic. A method more likely to give well-defined and not extensively degraded or decomposed proteins was introduced by HUISKAMP<sup>1</sup> and modified by CARTER AND HALL<sup>2</sup>. In this procedure the freshly pulped calf-thymus is extracted with water at 5° C for twenty-four to thirty-six hours, and the protein precipitated by the addition of sufficient salt to give 1% NaCl.

MIRSKY AND POLLISTER<sup>3, 4, 5</sup> have taken advantage of the fact that nucleoproteins will dissolve if the salt-percentage is raised to about 5%.

The protein prepared in this way will also dissolve in pure water, but according to MIRSKY AND POLLISTER its structure seems to change, as, if it is once dissolved in pure water and then redissolved in 1 M NaCl, the viscosity and the streaming birefringence have decreased when compared to the original solution in the same salt concentration. As a very small addition of salt will cause precipitation of the nucleoprotein from its solution in pure water, it is for most purposes much better to work with 1 M salt solution. This will also yield a higher concentration of nucleoprotein.

*Preparation:* Calf thymus is collected immediately after the animal has been killed. The fresh tissue is frozen with solid carbon dioxide and 200 g are ground in a mill together with additional solid carbon dioxide. 400 ml 1 M NaCl are then added to the powder of mixed protein and solid carbon dioxide. The solid carbon dioxide is allowed to evaporate in the cold storage room during the following night. The salt solution and the protein are now in the form of gel. This gel is then added to 2400 ml distilled water and the protein precipitates. The precipitate is thoroughly stirred to allow most of the blood to go into solution and is then collected and dissolved in 1 M NaCl. This new solution is centrifuged at a moderate speed in medium sized tubes, and the insoluble parts form an easily removable cake on the top of the solution. By the addition of six parts of distilled water a new threadlike precipitate is obtained. This is collected by twirling it around a glass rod and merely lifting it up. The precipitate is redissolved and the very viscous rather opalescent solution, is centrifuged at about 9500 r.p.m. for two hours. (The medium diameter of the centrifuge is 13 cm). This procedure is repeated until a total of five or six precipitations and redissolvings have been performed. All preparations are made in the cold storage room. Ample stirring is essential. In some cases the 1 M NaCl solution is buffered with a phosphate of 0.0125 M  $\text{NaH}_2\text{PO}_4$  plus 0.0125 M  $\text{Na}_2\text{HPO}_4$ , ionic strength 0.05. (This buffer is referred to as "phosphate buffer" on the following pages). The pH of the nucleoprotein-solutions will be very stable between 6.2 and 6.3. When no buffer is added the pH has a great tendency to drop to about 5.6.

The nucleoprotein prepared in this manner gave a white powder, when freeze-dried. For analysis it was dried at 105° C for 12 hours. The amount of phosphorus in the nucleoprotein-preparations was 3.7–3.8% and the nitrogen amounted to about 14.8%. (Preparations which had only been freeze-dried gave 3.4–3.5% P and 14.8% N). These values proved to be fairly constant after five precipitations and redissolvings. They

were also found to be constant if, when clearing the solutions of opalescence, the speed of the centrifuge was changed between the limits 27000 and 9500 r.p.m. (In both cases centrifugation was carried out for one hour). This is an interesting fact to be considered in the discussion of the degree of dispersity of the protein.

The analytical values are, however, a little too low, because of the adsorption of NaCl on the protein precipitate. If this factor is taken into account the values will be 3.8–3.9% P and 15.1% N. The sodium was determined as sodium sulphate and then weighed.

The nucleoprotein was checked for tryptophane with glyoxyl and copper sulphate plus sulphuric acid. The check was absolutely negative.

FEULGEN AND DISCHE reactions were positive.

All these facts agree very well with those given by MIRSKY AND POLLISTER.

#### ULTRACENTRIFUGATION

When the nucleoprotein, dissolved in 0.02 M NaCl, was centrifuged in the Svedberg ultracentrifuge, it showed a high degree of polydispersity. In 1 M NaCl the protein gave highly viscous solutions and then naturally the sedimentation constant was dependent on the concentration of the protein; giving one very sharp peak in the centrifugation diagram.

Only a few runs were made, because no striking differences from the values given by CARTER<sup>6</sup> were discovered. It must be stressed that due to the high viscosity no conclusions concerning the number of components can be drawn from the fact that only one peak was to be seen.

Two runs were made in the centrifuge at 40000 r.p.m. The sedimentation constants found were 12.9 for 0.62% nucleoprotein and 14.2 for 0.34% nucleoprotein. In both cases the solvent was 1 M NaCl and phosphate buffers (ionic strength 0.05) with  $p_H$  6.3.

#### ELECTROPHORESIS

When the nucleoprotein dissolved in neutral phosphate buffers (ionic strength 0.02) was investigated in the Tiselius' electrophoresis apparatus with the Svensson-Philpot optical system only one component was seen. This was negatively charged.

For one and the same preparation the mobility changes greatly with  $p_H$  and concentration. Different preparations also give great differences in mobility. To give the order of magnitude it may be mentioned that the mobility was found to be  $13.4 \cdot 10^{-5}$  cm<sup>2</sup>/volt sec at  $p_H$  6.24 for 0.27% nucleoprotein.

The investigation of nucleoprotein in 1 M NaCl in the electrophoresis apparatus was found to involve difficulties both in the performance of the experiments and their interpretation.

The compensation in the apparatus before starting each run must be done very slowly. If not, the boundary area will be extremely curved, because, due to the viscosity the middle of the area will be moved faster than the sides. The boundaries will then be very unstable.

Even if compensation is done with great care it is very difficult to avoid this effect on the descending side and in the following table only the values for the ascending side, moving towards the cathode, should be taken into consideration.

These values will give an average mobility of  $7.9 \cdot 10^{-5}$  cm<sup>2</sup>/volt sec of the main component. (See Table I). The voltage was in all runs, 50 V and the current about 43 mA. The runs were observed for about 18 hours.

TABLE I

ELECTROPHORESIS RUNS OF NUCLEOPROTEIN IN 1 M NaCl PLUS BUFFER AT 0.5 C. (BELOW  $p_H$  8 PHOSPHATE BUFFER, OVER  $p_H$  8 GLYCOCOLL-NaOH BUFFER. IONIC STRENGTH OF BUFFERS 0.1)

Prep. no.	Precipitated times	$p_H$		o/ % nucleoprotein	mobility $\cdot 10^5$ cm <sup>2</sup> /volt sec	
		solution	buffer		+	—
17	4	5.1	5.1	0.35	8.0	6.6
17	4	6.3	6.3	0.35	7.8	6.5
17	4	6.3	6.3	0.18	7.8	7.4
18	4	6.3	6.3	0.65	8.1; 6.5	7.8
17	5	6.3	6.3	0.36	8.0; 7.0; 6.4	6.9
18	5	6.3	6.3	0.50	7.9; 6.5	7.1
17	4	6.6	6.7	0.35	7.9	6.7
18	4	9.6	8.9	0.65	7.8	8.0
18	4	10.3	9.6	0.65	9.5	(7.9)

The average value for the mobility does not take into consideration the value for the solution with the highest  $p_H$  as this solution showed a very high opalescence and as the nucleoprotein solutions in this  $p_H$  region change their qualities. (See below).

The electrophoresis diagrams gave in all cases at least two and sometimes three peaks. The others were moving more slowly than the main peak, but were in some cases difficult to observe.

From the best experiment, that with preparation no. 17, precipitated 5 times (see Table I), it was found that the fastest component contributed to the area under the curve in the diagram with 50%, the slower with 16, and the slowest with 34%. In some cases there was also seen a very small peak in the diagram slowly moving backwards towards the anode.

Another difficulty was that some material diffused out of the cellophane bag during the dialysis. In these experiments, 10–13% of the nitrogen content went out in the buffer when dialysis was performed for 36 hours with stirring.

To make a more exact estimation of the extent of this loss 20 ml of a thymo-nucleoprotein in 1 M NaCl and phosphate buffer with ionic strength 0.05 ( $p_H$  6.3) were dialysed in a cellophane bag of the type used in the other experiments, against 40 ml of the same buffer and 1 M NaCl. The bag was rotated at a good speed. At the beginning the protein solution contains 1.09 mg N per ml. After 26 hours 13.8% of the nitrogen had passed out through the wall of the bag into the buffer, and after 98 hours, 17.0%. Samples for nitrogen determination were taken both outside and inside the bag. The dialysate was investigated for nucleic acid by the ultra-violet absorption method<sup>7</sup>, but it was found that all the nucleic acid had stayed in the bag.

The specific volume of the nucleoprotein in the dialysed solutions was 0.65.

Phosphate buffers were used for  $p_H$  lower than 8. For higher  $p_H$  glycoll-NaOH buffers were used.

While this work was being done van WINKLE AND FRANCE<sup>8</sup> published an investigation with the electrophoresis apparatus and the ultracentrifuge of a nucleoprotein prepared

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according to MIRSKY AND POLLISTER from rabbit liver. The mobilities found were of the same order of magnitude as those found for my preparations from calf thymus.

#### PRECIPITATION CURVES

An investigation was made of the solubility of nucleoprotein in NaCl-solutions of different molarities. The object of this investigation was to determine the form of the precipitation curve and see if under the usual conditions there were any changes in the percentage of nucleic acid in the precipitates.

As there is a marked solubility minimum at 0.14 M NaCl two separate series of determinations were made, one on each side of this minimum.

1. *Distilled water to 0.14 M NaCl.* A thoroughly washed protein precipitate was dissolved in distilled water. The solution was centrifuged for three hours at 9500 r.p.m. and contained 0.350 mg N/ml (Table II). 2 ml were taken from it, and 20 ml of accurately

TABLE II  
THE SOLUBILITY OF NUCLEOPROTEIN IN 0.00-0.14 M NaCl AT 3.8-4.2° C

M NaCl	Nitrogen mg/ml	Maximum extinc- tion (260 m $\mu$ )	Extinction N in mg/ml	PH
0.000	0.350	2.072	59	6.01
0.018	0.0276	1.758	64	5.61
0.036	0.0092	0.653	70	5.70
0.055	0.0080	0.520	65	5.70
0.073	0.0062	0.411	66	5.75
0.082	0.0060	0.392	65	5.70
0.091	0.0056	0.337	60	5.58
0.100	0.0048	0.341	71	5.58
0.109	0.0056	0.428	76	5.50
0.118	0.0048	0.331	69	5.62
0.141	0.0036	0.265	74	5.57

prepared salt solutions of different concentrations were added. Precipitates resembling small pieces of cotton wool immediately appeared in all mixtures, except that to which 0.020 M NaCl was added. It, however, showed opalescence. The mixtures were allowed to stand for three hours and the precipitate was then centrifuged down at 9500 r.p.m. The temperature during the whole procedure was between 3.8 and 4.2° C.

All solutions, together with the original one, were analysed for nitrogen by the Kjeldahl method. As nucleic acid shows a very specific absorption of light in the wavelength region of 260 m $\mu$ <sup>7</sup>, the absorption curves were determined for all solutions between 400 and 230 m $\mu$  in the Beckman spectrophotometer. The irrelevant absorption was very small, the absorption at 400 m $\mu$  being less than one per cent of the maximum absorption, and in the following table (No. 2) no correction has been made for it. The absorption measurements were made against the solvent.

The table values for N were obtained from the analysis of 5 ml of solution. As the values for the absorption of the original solution were somewhat high, they were measured after dilution at half the original protein concentration. The readings were then multiplied by two. The same method is used when necessary in all the following investigations. It was proved by separate experiments that BEER's law holds for these

preparations up to the highest extinction values which can possibly be determined directly and accurately with the apparatus, *i.e.*, an extinction of about 2 (see below).

From the quotient for the maximum absorption and the amount of nitrogen per ml, which is nearly constant over the whole curve, one can see that the nucleic acid is precipitated in parallel with the protein part. When judging the table values it must be understood that quotients of the nitrogen poor solutions are naturally somewhat uncertain.

2. *1 M to 0.14 M NaCl.* The original solution was centrifuged for  $1\frac{1}{2}$  hours at 3500 r.p.m. after being dissolved for the last time. (Earlier in the preparation the usual higher speed was used). To five ml of this nucleoprotein solution with 1 M NaCl and phosphate buffer (ionic strength 0.05) were quickly added different amounts of the same phosphate buffer. The mixtures were thoroughly shaken for a few minutes and then left for 5-6 hours. They were then centrifuged for one hour at 3500 r.p.m. All work was done in the cold storage room. The values measured are found in Table III.

TABLE III

THE SOLUBILITY OF NUCLEOPROTEIN IN 1.00-0.14 M NaCl PLUS PHOSPHATE BUFFER. (IONIC STRENGTH OF BUFFER 0.05). FIRST CURVE IN FIG. 1

Solution no.	M NaCl	Nitrogen mg/ml	$\lambda_{\max}$	Dilution for the absorption measurement	Observed maximum extinction	Theoretical extinction (corr. for dilution)	Extinction N in mg/ml
1	0.139	0.0036	265	1/1	0.093	0.093	—
2	0.139	0.0026	267	1/1	0.062	0.062	—
3	0.194	0.0024	265	1/1	0.042	0.042	18
4	0.292	0.0052	265	1/1	0.091	0.091	18
5	0.398	0.0732	260	2/7	0.714	0.250	34
6	0.422	0.1050	260	2/10	1.095	5.475	52
7	0.480	0.337	260	2/25	1.614	20.175	60
8	0.524	0.400	260	1/25	0.934	23.350	58
9	0.544	0.406	260	3/50	1.451	24.183	60
10	0.590	0.517	260	3/50	1.836	30.600	59
11	0.682	0.558	260	1/25	1.325	33.125	59
12	0.770	0.617	260	2/50	1.430	35.750	58
13	0.872	0.703	258	1/50	0.817	40.850	58
14	0.970	0.786	260	2/50	1.894	47.350	60

TABLE III (continued)

Solution no.	Temperature	pH	Type of precipitation (before centrifugation)
1	4.0-4.3	6.64	Immediate flocculant precipitation, clear liquid
2	4.0-4.3	6.64	" " " " "
3	4.0-4.3	6.59	" " " " "
4	4.0-4.3	6.51	" " " " "
5	4.0-4.3	6.45	" " " " "
6	4.0-4.3	6.49	Good precipitation, liquid somewhat opalescent
7	4.0-4.3	6.37	" " " " more "
8	1.2-1.6	6.35	" " " " "
9	4.0-4.3	6.32	Slight precipitation
10	4.0-4.3	6.30	Very little precipitation
11	1.2-1.6	6.28	Opalescence
12	4.0-4.3	6.28	"
13	1.2-1.6	6.34	"
14	4.0-4.3	6.27	"



It is seen from the values for the temperature in the table that the precipitations were made in two different series.

It may also be stressed that in the cases where the maximum absorption did not occur at 260  $m\mu$ , the difference between the maximum value and the reading at 260  $m\mu$  was very small; for solution no. 13, well within the limits of error.

Since the protein precipitate used for preparing the original solution cannot be dried completely, the salt molarity of the original solution will be somewhat diminished. For this reason, the values of the salt concentrations have an error of  $\pm 0.01$  M, but the relative values between the different solutions are correct to the second decimal.

A second curve was made in approximately the same manner as the first one (see Table IV). The difference is that another preparation was made and that the precipita-

TABLE IV

THE SOLUBILITY OF NUCLEOPROTEIN IN 1.00–0.14 M NaCl PLUS PHOSPHATE BUFFER AT 1.8° C.  
PH 6.60–6.25. IONIC STRENGTH OF BUFFER 0.05. SECOND CURVE IN FIG. 1

M NaCl	Nitrogen mg/ml	Extinction (corr. for dilution)	Extinction N in mg/ml
0.243	0.005	0.054	—
0.340	0.017	0.229	—
0.399	0.057	1.680	29
0.423	0.130	3.930	30
0.468	0.200	10.85	54
0.483	0.205	11.12	54
0.506	0.231	12.75	55
0.534	0.257	14.22	55
0.563	0.278	15.44	56
0.584	0.321	17.20	54
0.683	0.380	21.40	56
0.722	0.400	22.30	56
0.795	0.452	25.20	56
0.825	0.481	25.80	54
0.892	0.509	27.86	55
0.970	0.578	31.68	55

tions were allowed to stand for 18 hours before centrifugation for one hour at 9500 r.p.m. The temperature was 1.8° C and  $p_H$  between 6.60 and 6.25, with decreasing values for increasing salt concentrations.

For all samples in both series the absorption at 400  $m\mu$  was less than 1.5% of the maximum absorption and also about the same for all solutions. No correction for the irrelevant absorption has therefore been made.

From the values it can be seen that the nucleic acid precipitates in parallel with the protein. It can also be observed that both curves in Fig. 1 show typical sudden changes. The nucleoprotein precipitates from different parts of these curves will be investigated later to determine if possible whether the form of the curves is due to a different type of nucleoproteins, to which the electrophoresis investigations hint, or to changes in the character of the highly viscous solution.

It can however be said with certainty that the precipitation by dilution from 1 M to 0.14 M NaCl, made in the preparation of nucleoprotein, approaches 100%. Less than 0.2% of the nitrogen content and extinction coefficient of the original solution remain in

the solution which contains physiological saline solution. From the two experiments with 0.14 M NaCl, one centrifuged and the other decanted, it can be seen that this makes very little difference.

To control the degree of precipitation 14 samples were taken from the same nucleoprotein solution in 1 M NaCl, half of which were precipitated as above. That is to say the solutions were merely allowed to stand in the coldroom for a number of hours after dilution to different NaCl concentrations and the precipitates then centrifuged down.

The remaining seven samples were diluted to the same NaCl concentrations as the others and then shaken for twenty hours in an icewater bath.

The precipitates in all samples were centrifuged down at 9500 r.p.m. under the same conditions.

Their nitrogen content was determined. The differences in nitrogen found between the two differently treated types of solutions were very small, and were well within the limits of error.

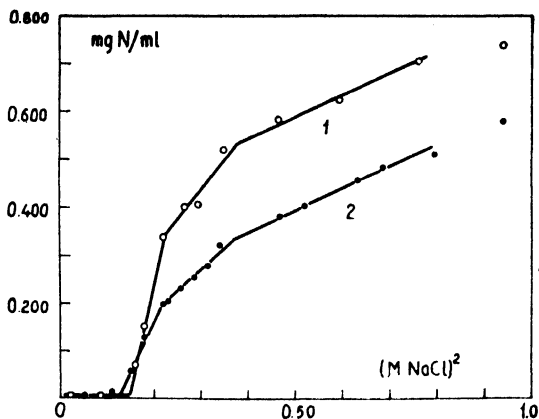


Fig. 1. Amount of nitrogen in mg/ml plotted against the square of the molarity of NaCl in two series, where a solution of nucleoprotein in 1 M NaCl plus phosphate buffer (ionic strength 0.05) was diluted to different NaCl concentrations. The two solutions have been prepared in somewhat different ways (See also Tables III and IV).

#### VISCOSITY

Measurements of viscosity have been made earlier by among others, CARTER<sup>9</sup> and CARTER AND HALL<sup>2</sup> in preparations made according to HUISKAMP and with dilute phosphate buffer as a solvent, with or without the addition of NaCl. The protein content was varied and the temperature was kept either at 0.7 or 25° C.

VON EULER AND FISHER<sup>10</sup> have prepared cell nuclei by a method according to DOUNCE, dissolved them in 1 M NaCl and measured viscosity with variations in  $p_H$  and protein concentration.

GREENSTEIN AND JENRETTE<sup>11</sup> have prepared nucleoprotein from calf thymus and cow liver. They precipitated the protein at  $p_H$  4.2 in an 0.8 M salt solution and washed it with KCl-acetate buffer. The protein was dissolved in "fairly strong" NaOH, to which was afterwards added guanidine — HCl or urea in different concentrations. This addition resulted in a great decrease in the very high viscosity.

GREENSTEIN AND JENRETTE<sup>12</sup> have also shown, that the viscosities of solutions of pure nucleic acid are very sensitive to salt additions and especially to the guanidinium-ion. Urea also has a strong effect.

The purpose of the following viscosity measurements was to determine whether or not the effect found when salt is added to nucleic acid solutions also exists for additions of salt to solutions of mildly prepared nucleoprotein.

### I. Measurements on Solutions with a $p_H$ of about 6.3

This investigation was carried out partly on protein, which had already been dissolved in 1 M NaCl, to which had been added solutions of NaCl, NaI, guanidine-HCl, guanidine-HNO<sub>3</sub> and urea, and partly on protein which had been prepared and precipitated in the usual manner and then directly dissolved in solutions of NaI, and guanidine-HCl. Viscosity measurements on these solutions were made with different protein concentrations. In urea practically no nucleoprotein dissolved.

The measurements were carried out in an Ostwald viscosimeter, which was placed in a well insulated thermostat filled with ice water.

The different measurements, obtained after the addition of different salts are given in Table V. "Relative viscosity" in the table is obtained directly by dividing the time for the outflow of the nucleoprotein solution by the time for the solvent in each case.

TABLE V

VISCOSITY CHANGES AT 0° C IN NUCLEOPROTEIN SOLUTION CONTAINING 0.165 mg N/ml DISSOLVED IN 1 M NaCl PLUS PHOSPHATE BUFFER (IONIC STRENGTH 0.05) WITH ADDITION OF DIFFERENT SALTS

Added salt solution Final conc.	Rel. visc. of protein- solution and solvent + added salt	pH
2 M urea	2.98	6.3
2 M NaCl	3.03	6.2
Saturated gu-HNO <sub>3</sub>	3.03	5.8
About 1.8 M gu-HCl	3.13	5.9
About 1.8 M gu-HCl	3.14	6.0
2 M gu-HCl	3.12	6.2
2 M NaI	3.05	6.2
No salt added	2.81	6.3

From the table it is quite clear that there is no change in the viscosity by the addition of guanidinium-ion, urea or NaI to nucleoprotein solutions in 1 M NaCl slightly buffered at  $p_H$  6.3. This is in striking contrast to the large decrease in viscosity obtained by the addition of these salts to nucleic acid solutions.

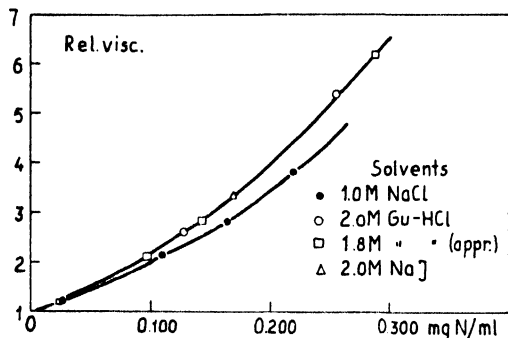


Fig. 2. Relative viscosity at 0° C for nucleoprotein in different salt solutions with  $p_H$  between 6.2 and 6.3

The result of directly dissolving nucleoprotein freshly precipitated in three different salt solutions will best be understood from the curve in Fig. 2.

The nucleoprotein itself is from the same preparation as that in the first experiment above.

It can be stated that any decrease in the viscosity of nucleoprotein solutions, when changing from NaCl to guanidine-HCl or NaI as the solvent is not observed.

In two preparations the possible change of viscosity with time was investigated (see Table VIa and b). The solutions were kept at 0° C the whole time. Time is measured from the start of the last dissolving.

When stirring, the viscosity, as seen from the table changed a little in the second decimal. The slight decrease probably depended on the fact that water condensed in the cold solution when it was allowed to stand exposed to the air for a while. No thixotropic effect seems, however, to exist.

TABLE VIa

INFLUENCE OF TIME ON VISCOSITY AT 0° C OF NUCLEOPROTEIN (PREPARATION Th 13) IN 1 M NaCl PLUS PHOSPHATE BUFFER (IONIC STRENGTH 0.05). THE PROTEIN SOLUTION STORED AT 0° C

Time in days	Rel. visc.	
1	2.81	pH 6.3-6.2 0.165 mg N/ml
3	2.83	
8	2.89	
9	2.91	

TABLE VIb

INFLUENCE OF TIME ON VISCOSITY AT 0° C OF NUCLEOPROTEIN (PREPARATION Th 12) IN 1 M NaCl PLUS PHOSPHATE BUFFER (IONIC STRENGTH 0.05). THE PROTEIN SOLUTION STORED AT 0° C

Time in days	Rel. visc.	
9	2.88	pH 6.3-6.2 0.24 mg N/ml
11	2.93	
19	3.06	
30	3.27	
33	3.27	
Stirring for 100 min	3.22	
.. .. 180 min	3.20	

For the most part, one can say that the viscosity of these nucleoprotein solutions remains constant for several weeks if the solutions are correctly stored. Depolymerization does not seem to take place. It must however, be stressed that after a longer time the solutions seem to undergo essential changes, even with the viscosity unchanged, as the tendency to precipitate on dilution is diminished.

## II. Measurements on Solutions of $p_H$ 6.3 and higher

It was found that fresh nucleoprotein dissolved in both 0.05 M NaOH or in 1 M NaCl gave high viscosities. Relative viscosity was about 2.9 in both cases. If salt was added to the solution of protein in base, the viscosity decreased very rapidly down to about 1.1. The same viscosity value was obtained if NaOH was added to a solution of nucleoprotein in 1 M NaCl. The decrease, however, is somewhat slower in the latter case.

After these first experiments a series of measurements of additions of NaOH to nucleoprotein in 1 M NaCl were made. Small samples of the protein solution were taken, and solvent and 0.100 M NaOH were added to each sample, giving the same protein concentration, the same 1 M NaCl, but a different amount of NaOH.

The solutions were kept for about 24 hours in the refrigerator after the addition of NaOH. (Except the one marked with an X in the curve Fig. 3, which was kept at room temperature).

The increase in viscosity, which preceded the final decrease, is remarkable. The curve in this region is dotted to show that the values both for  $p_H$  and viscosity are rather unstable. The time for outflow in the viscosimeter decreased by as much as 10 seconds from one reading to another. The  $p_H$  before and after three readings usually differed by one or two tenths. The other parts of the curve gave reproducible and stable values.

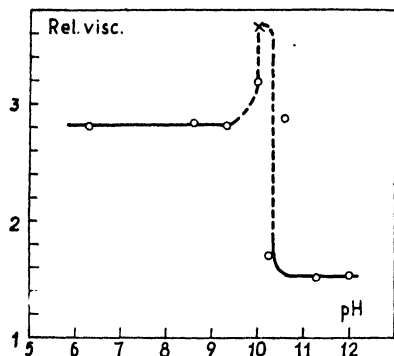


Fig. 3. Relative viscosity at 0° C of nucleoprotein in 1 M NaCl at different  $p_H$ . Different amounts of 0.100 M NaOH have been added 24 hours before the measurements

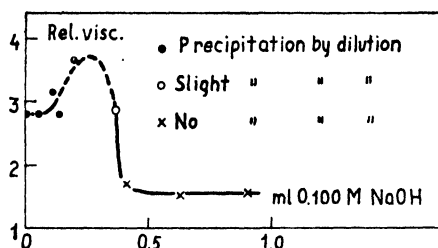


Fig. 4. Relative viscosity at 0° C of a nucleoprotein (same as in Fig. 3) in 1 M NaCl after adding different amounts of 0.100 M NaOH. The measurements were made 24 hours after the addition of the base

The definite change in viscosity seems to come after an addition of  $4 \cdot 10^{-5}$  mol NaOH to 10.7 mg nucleoprotein, calculated on basis of a N-content of 15.1%.

It has also been indicated on the curve (Fig. 4) that the nucleoprotein solutions lose their ability to precipitate on dilution at the same time that their viscosities tend to become dependent on salt concentration. Tests of precipitability were made by adding 5 ml of distilled water to 1 ml of each solution. The precipitates were centrifuged down at 9500 r.p.m. for half an hour. 4 ml of the solutions were investigated for N according to KJELDAHL (see Table VII).

TABLE VII  
AMOUNT OF NITROGEN LEFT IN THE SOLUTION  
AFTER PRECIPITATION BY DILUTION AT DIFFERENT  
PH VALUES

mg N/4 ml	ml 0.100 M NaOH added to the original samples
0.015	0.00
0.011	0.05
0.018	0.11
0.010	0.14
0.010	0.20
0.084	0.37
0.095	0.42
0.109	0.63
0.119	0.90

#### THE ULTRAVIOLET ABSORPTION

##### I. Nucleic Acid Content from Percentage P and Light Absorption

As has been already stated the nucleoprotein solutions gave the typical curve for

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ultra-violet absorption which was given by CASPERSSON<sup>7</sup> and others. This curve, with the maximum about 258–260  $m\mu$ , was of the same type for dried preparations dissolved in dilute NaOH and for fresh nucleoprotein dissolved in 1 M NaCl.

The fact that the nucleoprotein followed BEER's law was proved with a preparation first freeze-dried and then dried in vacuum over phosphorus pentoxide at 90° C for 72 hours and then dissolved in 0.100 M NaOH. The absorption in ultra-violet light at 260  $m\mu$  was then measured for four different concentrations of nucleoprotein: 0.0059 mg N/ml gave an absorption of 0.460, 0.0118 mg N/ml gave 0.940, 0.0148 mg N/ml gave 1.180 and 0.0178 mg N/ml gave 1.415. For all four measurements this means a quotient of 80 between extinction and mg N/ml.

This value gives a percentage of nucleic acid of about 44%, if compared with absorption measurements made by B. DRAKE (*unpubl.*) on pure nucleic acid, (the same preparation as used by BJÖRNESJÖ AND THEORELL<sup>13</sup>). The P-content of the nucleoprotein preparations described in this paper was 3.8–3.9% which gives a nucleic acid content of only 38–39%, if calculated on the basis of the value from LEVENE's formula — 9.91% P for thymus nucleic acid. It is, however, assumed nowadays that this formula gives too high a phosphorus percentage.

The nucleic acid used as comparison gives 7.6% P as tetrasodium salt. The highest percentages yet found in tetrasodium nucleate preparations according to HAMMERSTEN are 8.8 and 8.4 given by GREENSTEIN<sup>14</sup>. These values give a nucleic acid content, calculated from the phosphorus content, closer to that given by estimation from absorption curves. GULLAND, JORDAN, AND THRELFALL<sup>15</sup> have, however, with another preparative method obtained tetrasodium nucleate with a phosphorus content quite near to the value calculated from LEVENE's formula. It thus seems clear that all the phosphorus in the nucleoprotein preparations used here comes from the nucleic acid.

## II. The Influence of $p_H$ on the Absorption Curve

The calculation above is valid for nucleoprotein dried in the way above mentioned and then dissolved in dilute NaOH. If the absorption is measured on fresh preparations in 1 M NaCl, there will be a lower quotient between the maximum extinction at 260  $m\mu$  and the nitrogen content measured in mg N/ml.

In the three solubility curves quotients about 59 and 55 are found for protein in 1 M NaCl at the beginning, and between 65–76 for protein originally dissolved in distilled water. In the first case the lower values belong to the curve where the precipitate was centrifuged down at the highest speed. In the second case the first and lower value is the most reliable, as the N-determinations become more uncertain for solutions near 0.14 M NaCl, which give higher quotients.

Some control experiments were carried out. It was first found that if from the same fresh precipitate one half was redissolved in 1 M NaCl and phosphate buffer ( $p_H$  6.3; ionic strength 0.05) and the other half dried as above and then dissolved in dilute NaOH, the quotient between extinction at 260  $m\mu$  and N/ml was for the first solution only 59 or 72% of the same quotient for the second solution, which gave the value 82.

In Fig. 5, curve number 1, a typical curve is seen, for the light absorption of nucleoprotein in 1 M NaCl at a  $p_H$  of about 6.3, obtained with the Beckman apparatus and measured against the solvent. The extinction per mg N/ml is 56.

It was now found that if diluted NaOH was added to such a nucleoprotein solution the quotient between extinction and mg N/ml rose to 74. To compare the two absorption

curves the solution of nucleoprotein giving curve number 1 was diluted with 1 part diluted NaOH to 3 parts of solution and curve number 2 in Fig. 5 was obtained. The NaCl content is kept constant at 1 M.

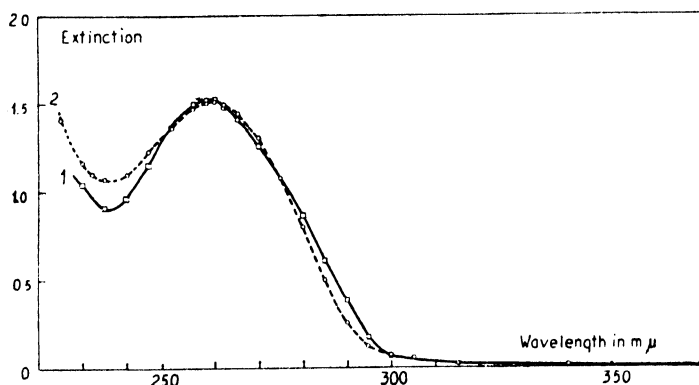


Fig. 5. Light absorption for nucleoprotein in 1 M NaCl (0.0271 mg N/ml) is shown in curve no. 1. The dotted line, curve no. 2, gives the values if 1 vol. part of 0.10 M NaOH + 1 M NaCl is added to 3 parts of the first solution

To avoid any effects of the added substances, the dilution is done parallel in the two BECKMAN cells.

With this more gentle treatment the light absorption of neutral nucleoprotein is only 75% of the basic one.

It can be concluded from what has been said above, that the absorption increases at 260 mμ if the nucleoprotein is treated with NaOH. If the nucleoprotein is treated more roughly, that is to say, dried and then redissolved in NaOH, the absorption increases still further. HOLIDAY<sup>16</sup>, HEYROTH AND LOOFBOUROW<sup>17</sup> have shown that some purines and pyrimidines increase their ultra-violet absorption at higher  $p_H$  values. Concerning the nucleoprotein it may also be pointed out that the change in absorption occurs at the same  $p_H$  at which the viscosity falls from the addition of salts.

I wish to thank my teacher, Professor A. TISELIUS, who introduced me to this field of work, for all his kind advice and helpful discussions. I also wish to thank Professor T. SVEDBERG for allowing me to perform this work at the Institute of Physical Chemistry in Uppsala.

#### SUMMARY

Calf thymus nucleoprotein has been prepared according to MIRSKY AND POLLISTER giving a content of 3.8–3.9% phosphorus and 15.1% nitrogen.

Ultracentrifugation and electrophoresis have been carried out on the nucleoprotein dissolved both in 1 M NaCl and in distilled water. The nucleoprotein in 1 M NaCl and phosphate buffer shows three components, the main one having a mobility of  $7.9 \cdot 10^{-5}$  cm<sup>2</sup>/volt sec.

When nucleoprotein dissolved in 1 M NaCl was precipitated through dilution, the nucleic acid precipitated in parallel with the protein. From 1 M down to about 0.35 M NaCl the precipitation curve is composed of three parts, giving three approximately straight lines if the nucleoprotein is plotted against the square of the salt molarity. Between 0.30 and 0.05 M NaCl practically all the nucleoprotein is precipitated. The nucleoprotein dissolves in distilled water.

From the measurements of light absorption at 260 mμ it was found that about 44% of the nucleoprotein is nucleic acid. This agrees with the percentage of phosphorus, showing that all phosphorus comes from the acid.

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The nucleoprotein has a high viscosity both in 1 M NaCl and in dilute NaOH. But if NaOH is added to the former type of solution the viscosity drops at a pH about 10.5. The viscosity also drops if salts are added to the solution of nucleoprotein in NaOH.

No decrease in viscosity was obtained, when guanidinium-ion or urea was added to the solution of nucleoprotein in 1 M NaCl.

The viscosity does not decrease with time if the nucleoprotein solution is stored in the cold.

The maximal extinction of light at 260  $m\mu$  increases if the nucleoprotein is treated with NaOH. The position of the extinction maximum does not change.

## RÉSUMÉ

La nucléoprotéine du thymus de veau a été préparée d'après la méthode de MIRSKY ET POLLISTER. La substance contient 3.8 à 3.9 % de phosphore, et 15.1 % d'azote. La nucléoprotéine dissoute soit dans NaCl 1 M soit dans de l'eau distillée, a été soumise à l'ultracentrifugation et à l'électrophorèse. Dans NaCl 1 M tamponné par des phosphates, la nucléoprotéine est formée de trois constituants dont le principal a une mobilité de  $7.9 \cdot 10^{-5}$  cm<sup>2</sup>/volt sec.

Lorsque la nucléoprotéine dissoute dans NaCl 1 M est précipitée par dilution, l'acide nucléique précipite parallèlement à la protéine. Pour des concentrations de NaCl allant de 1 M à environ 0.35 M, la courbe de précipitation comporte trois parties, s'exprimant approximativement par des lignes droites si la teneur en nucléoprotéine est exprimée en fonction du carré de la molarité du sel. Lorsque la concentration en NaCl est entre 0.30 et 0.05 M, pratiquement toute la nucléoprotéine est précipitée. La nucléoprotéine se dissout dans l'eau distillée.

La mesure de l'absorption lumineuse à 260  $m\mu$  montre que 44 % de la nucléoprotéine consiste en acide nucléique. Ceci correspond à la teneur en phosphore, ce qui montre que tout le phosphore appartient à l'acide nucléique.

La nucléoprotéine possède une viscosité élevée, aussi bien dans NaCl 1 M que dans la soude diluée. Mais si l'on ajoute de la soude à la solution dans NaCl, la viscosité diminue à un pH voisin de 10.5. La viscosité diminue également si on ajoute des sels à la solution de nucléoprotéine dans NaOH.

La viscosité ne diminue pas lorsque l'on ajoute l'ion guanidinium ou de l'urée à la solution de nucléoprotéine dans NaCl 1 M.

La viscosité ne diminue pas avec le temps lorsque la solution de nucléoprotéine est conservée au froid.

L'extinction maximum de la lumière à 260  $m\mu$  s'accroît si la nucléoprotéine est traitée par NaOH, mais la position du maximum d'extinction reste la même.

## ZUSAMMENFASSUNG

Kalbthymusnukleoprotein wurde nach MIRSKY UND POLLISTER mit einem Phosphorgehalt von 3.8–3.9 % und einem Stickstoffgehalt von 15.1 % bereitet.

Mit Lösungen des Nukleoproteins, sowohl in 1 M NaCl als in destilliertem Wasser wurden Ultrazentrifugierungs- und Elektrophoresemessungen ausgeführt. In 1 M NaCl und Phosphatpuffer zeigt das Nukleoprotein drei Komponenten, von denen die Hauptkomponente eine Beweglichkeit von  $7.9 \cdot 10^{-5}$  cm<sup>2</sup>/Volt sec. hat.

Bei Fällung von Nukleoprotein, das in 1 M NaCl gelöst war, durch Verdünnen schlug die Nukleinsäure parallel mit dem Eiweiss nieder. Von 1 M ab bis zu ungefähr 0.35 M NaCl ist die Präzipitationskurve aus drei Teilen zusammengesetzt, die drei angenähert gerade Linien ergeben, wenn der Nukleoproteingehalt gegen das Quadrat der Salzmolarität aufgetragen wird. Zwischen 0.30 und 0.05 M NaCl wird praktisch das gesamte Nukleoprotein gefällt. Das Nukleoprotein löst sich in destilliertem Wasser.

Aus Messungen der Lichtabsorption bei 260  $\mu m$  wurde festgestellt, dass ungefähr 44 % des Nukleoproteins Nukleinsäure ist. Dieses Ergebnis stimmt mit dem Phosphorgehalt überein. Es zeigt sich also, dass der gesamte Phosphor aus der Nukleinsäure stammt.

Das Nukleoprotein hat sowohl in 1 M NaCl wie auch in verdünnter NaOH eine hohe Viskosität. Wenn jedoch zu ersterer Lösung NaOH zugefügt wird, fällt der Viskositätswert bei einem pH von ungefähr 10.5. Die Viskosität wird auch erniedrigt, wenn zu der Lösung des Nukleoproteins in NaOH Salze hinzugefügt werden.

Bei Zugabe von Guanidinium-ionen oder Harnstoff zu der Nukleoproteinlösung in 1 M NaCl wurde keine Viskositätsverringerung erhalten.

Die Viskosität nimmt im Laufe der Zeit nicht ab, wenn die Nukleoproteinlösung kalt aufgehoben wird.

Die maximale Lichtextinktion bei 260  $m\mu$  nimmt bei Behandeln des Nukleoproteins mit NaOH zu. Die Lage des Extinktionsmaximums ändert sich dabei nicht.

References p. 116.



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# DEPENDENCE OF THE $p_H$ -OPTIMUM OF THE PHOSPHOMONOESTERASE I ON THE SUBSTRATE CONCENTRATION AND ON INHIBITORS AND ACTIVATORS

by

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## INTRODUCTION

In previous publications we pointed to the bearing of  $p_H$  activity curves on the determination of phosphatase in extracts of organs and serum<sup>1, 2, 3</sup>. We demonstrated that small differences in  $p_H$  might cause great differences in activity. Hence for the accurate determination of phosphatase it is better to establish activity curves than to content oneself with one determination at optimum  $p_H$ . Moreover these curves give an indication of the presence of isodynamic phosphatases. For the various organs we found specific activity curves. Fig. 1 shows the  $p_H$  curve for the kidney, Fig. 2 that for the intestine of the rat.

It is notable that the optimum  $p_H$  for phosphomonoesterase I (the so-called alkaline phosphatase) is so high. Although we are rather in the dark regarding the value of the  $p_H$  in the living cell or part of the cell, yet a  $p_H$  of 9.8 must be considered to be unphysiological. Further it is remarkable that the ferment deploys so little activity at more physiological  $p_H$  values.

The presence of accompanying substances can influence the optimum  $p_H$  of an enzyme reaction. This is the reason that identical  $p_H$  optima are not always found for one and the same ferment in unpurified extracts of various organs. For various organs these differences are very slight as far as the alkaline phosphatase is concerned. Phosphomonoesterase I in intestine extract has its maximum action at  $p_H = 9.6$ ; in kidney extract at  $p_H = 9.9$ . FRANKENTHAL<sup>4</sup> has found that an acid phosphatase from sarcomas shows another optimum when  $Mg^{++}$  and  $Mn^{++}$  ions are present than when these ions are absent. We have now investigated the influence of various substances on the alkaline phosphatase.

When ferments are purified, which evidently causes many accompanying substances to be removed, it sometimes happens that the optimal action of the purified

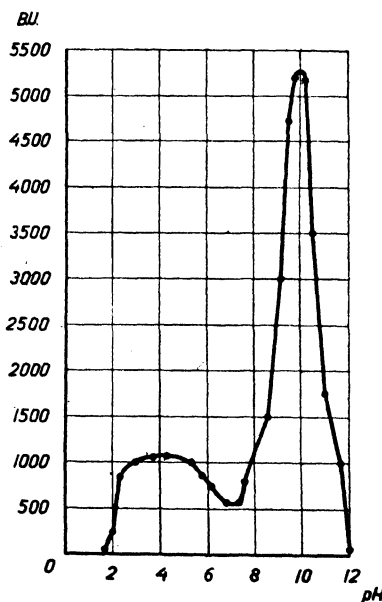


Fig. 1.  $p_H$ -activity-curve of the phosphatase(s) of the kidney of the rat. (B.U. = BODANSKY UNIT)

enzyme is found at another  $p_H$  than that of the initial material. FROMAGEOT<sup>5</sup> describes this phenomenon for glucosulphatase. We have subjected phosphomonoesterase I to a purification, examining whether also in this case the  $p_H$  optimum changed.

The  $p_H$  optimum is dependent on the substrate used. DELORY AND KING<sup>6</sup> stated that the optimum  $p_H$  for phosphomonoesterase I is higher for phosphoric acid esters with a low degree of dissociation than for esters with a higher degree of dissociation.

With one particular substrate the optimum  $p_H$  of a ferment reaction sometimes depends on the substrate concentration.

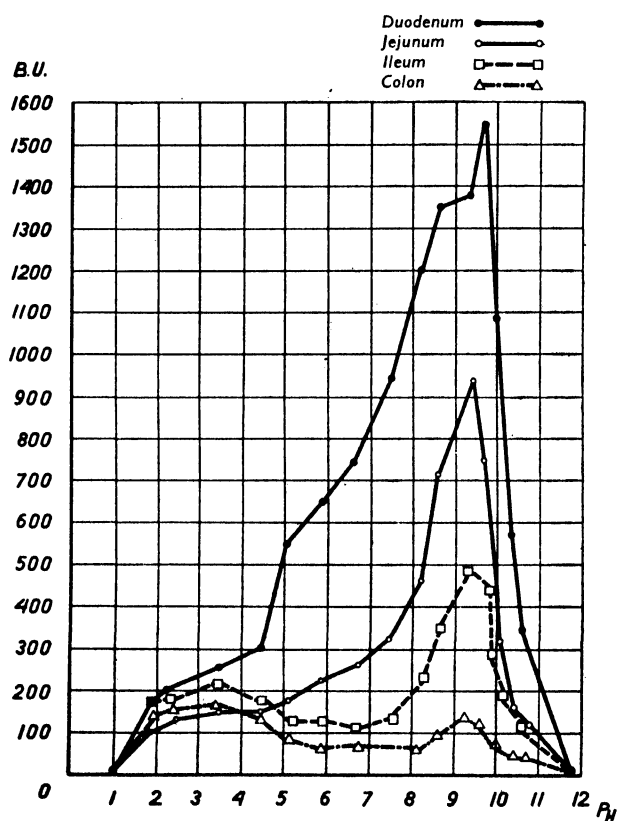


Fig. 2.  $p_H$  activity-curve of the phosphatase(s) of the intestine of the rat

It is assumed that the enzyme reaction passes through various phases. First there is a combination of enzyme and substrate, forming a complex.



Then there is a disintegration:



Both reactions depend to a different extent on the  $p_H$ . Finally the equilibrium



is also in many cases influenced by the  $p_H$ .

VAN SLYKE<sup>7</sup> assumes that with a low concentration of the substrate reaction 1

predominates. With a high concentration of the substrate the enzyme is fairly constantly combined with substrate molecules and reaction 2 predominates. For urease and arginase the optimum p<sub>H</sub> was shown to be dependent on the concentration of the substrate<sup>7</sup>. We have examined whether with the alkaline phosphatase the optimum also varies with the concentration of the substrate.

## EXPERIMENTAL PART

### I. METHODS OF PHOSPHATASE DETERMINATION<sup>1, 2, 3</sup>

For these we refer to our previous publications.  
Na- $\beta$ -glycerophosphate served as a substrate.

### II. INFLUENCE OF ACCOMPANYING SUBSTANCES IN THE EXTRACTS

For the preparation of extracts of organs, as this was done for determining activity curves<sup>1</sup>, the organ is ground up with powdered quartz, consequently the environment in which the phosphatase reaction takes place may be quite different from that of the cells which contain the phosphatase in the living organ. The low-molecular substances are removed from the extract by dialysis. We could confirm the observation described by ALBERS<sup>8, 9</sup> that in the kidney extract a substance occurs which inhibits the phosphatase. In intestine extracts activity was the same before and after dialysis. The optimum p<sub>H</sub> did not vary. The extracts were dialysed in a collodion bag for 24 hours against running tapwater and afterwards once more against distilled water till no more PO<sub>4</sub>''' ions were demonstrably present.

TABLE I

Organ extract	P.U.*	p <sub>H</sub> optimum
Kidney before dialysis	19	9.9
Kidney after dialysis	25	9.9
Intestine before dialysis	15	9.6
Intestine after dialysis	16	9.6

### III. PURIFICATION OF PHOSPHOMONOESTERASE I

The organ extract was brought to p<sub>H</sub> = 4.5 with the aid of 1 n. acetic acid. Per ml of extract 25 mg kaolin was added and the mixture shaken. After 15 minutes centrifugation took place and the centrifugate was poured off. The precipitate was eluted with 33% ethanol, which had been brought to p<sub>H</sub> = 9 with ammonia. After centrifugation the p<sub>H</sub> of the centrifugate was reduced to 7 and ethanol was added drop by drop, the centrifugate being stirred continuously till the ethanol concentration amounted to 70%. The result was a precipitate from which phosphomonoesterase I could be extracted at p<sub>H</sub> 8.7. A large proportion, however, remains insoluble (we found that when precipitation takes place at -10° C readily soluble preparations are obtained.\*\*

\* P.U. = Phosphatase-Unit (ALBERS<sup>8</sup>)

\*\* To be published shortly

It is seen that at low substrate concentration the curve shifts to the neutral region. That this is also the case for the phosphatase of the intestine is proved by Fig. 7.

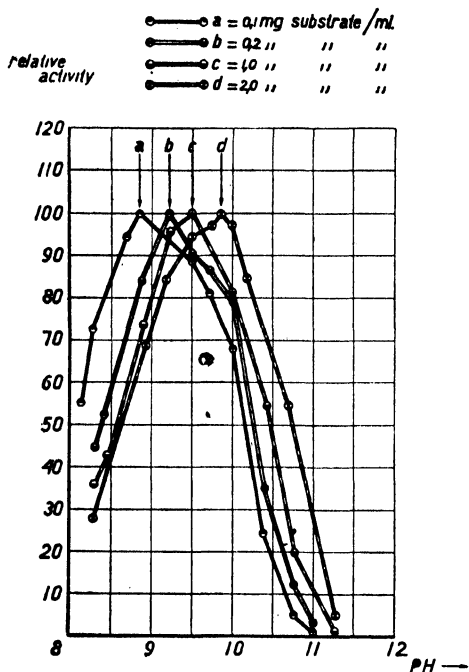


Fig. 6. Kidney of the rat pH-activity-curves of phosphomonoesterase I with different substrate concentrations

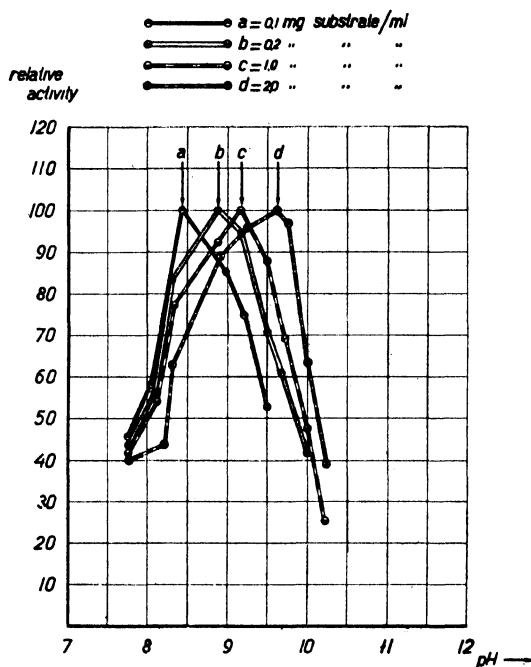


Fig. 7. Intestine of the rat pH-activity-curves of phosphomonoesterase I with different substrate concentrations

### CONCLUSION

When determining  $p_H$  activity curves for phosphomonoesterase I we always choose a substrate concentration of 4–6 mg/ml (Na- $\beta$ -glycerophosphate) in the reaction liquid (buffer + enzyme solution + substrate solution). Then we are on the horizontal part of the substrate concentration curve, which is desirable for the determination; the rate of reaction has then become independent of the substrate concentration. The hydrolytic reaction turns out to pass through several phases. At lower substrate concentration the  $p_H$  curve shifts to the neutral region. In the living cell, where substances are continuously arriving or from which they are removed, the substrate concentrations will be low. In the cells the optimum  $p_H$  for phosphomonoesterase I will be lower than would follow from the activity curve.

We wish to express our thanks to the ROCKEFELLER FOUNDATION for helping us to carry out this research.

## SUMMARY

1. The fact is pointed out that according to the p<sub>H</sub> activity-curve of phosphomonoesterase I from animal organs the ferment is so highly active at an unphysiological p<sub>H</sub> and so little active in the physiological p<sub>H</sub> region.

2. For the alkaline phosphatase it was shown that the substrate concentration influences the position of the p<sub>H</sub>-activity curve. At a low substrate concentration the curve shifts to the neutral region. It is expounded that in the cell the substrate concentrations will be low. For the determination of the p<sub>H</sub>-activity curves the substrate concentration chosen is generally such as will not affect the rate of reaction. These are comparatively high concentrations, so that inferences about the optimum p<sub>H</sub> for the phosphatase activity in the cell cannot be drawn from them.

3. By dialysis of kidney extracts it was possible to remove an inhibitory substance. Before and after dialysis the optimum is found at the same p<sub>H</sub>.

4. Phosphomonoesterase I could be adsorbed to kaolin and could afterwards be eluted again. Subsequently the enzyme is precipitated with ethanol. The preparations purified in this way contain no other phosphomonoesterases; however, they do contain pyrophosphatase I. By adsorption to diphenylmethanol and a subsequent elution phosphomonoesterase I could be freed from pyrophosphatase. The purification did not affect the position of the optimum p<sub>H</sub>.

5. The influence of a number of cations and anions on the situation of the optimum p<sub>H</sub> of the alkaline phosphatase was tested. They were shown to have practically no influence.

## RÉSUMÉ

1. L'auteur souligne le fait que, d'après la courbe d'activité en fonction du p<sub>H</sub>, de la phosphomonoestérase I des organes animaux, l'enzyme est extrêmement active à des p<sub>H</sub> non-physiologiques et, au contraire, fort peu aux p<sub>H</sub> physiologiques.

2. La concentration du substrat influence la position du p<sub>H</sub> optimum de la phosphatase alcaline. Pour une faible concentration en substrat, la courbe d'activité en fonction du p<sub>H</sub> est décalée vers la neutralité. Il est vraisemblable que, dans la cellule, la concentration en substrat est faible. La détermination des courbes d'activité en fonction du p<sub>H</sub> se fait habituellement en présence de concentrations en substrat telles qu'elles n'influencent pas la vitesse de la réaction. Ce sont là des concentrations relativement élevées; aussi ne peut-on rien en déduire quant au p<sub>H</sub> optimum des phosphatases dans la cellule.

3. La dialyse d'extraits de rein permet d'éliminer une substance inhibitrice. Le p<sub>H</sub> optimum reste le même avant et après dialyse.

4. La phosphomonoestérase I peut être adsorbée sur kaolin, puis en être éluée. L'enzyme est ensuite précipitée par l'éthanol. Une telle préparation ne contient pas d'autre phosphomonoestérase, mais contient encore la pyrophosphatase I. Celle-ci peut être éliminée par adsorption de la phosphomonoestérase I sur le diphenyl méthanol, suivie d'une élution. Cette purification ne modifie pas le p<sub>H</sub> optimum.

5. L'influence d'une série de cations et d'anions sur le p<sub>H</sub> optimum de la phosphatase alcaline s'est révélée nulle.

## ZUSAMMENFASSUNG

1. Die Kurve der Aktivität der Phosphomonoesterase I in Abhängigkeit vom p<sub>H</sub> lässt erkennen, dass das Enzym bei nicht physiologischen p<sub>H</sub>-Werten sehr aktiv, bei physiologischen Werten dagegen sehr wenig aktiv ist.

2. Für die alkalische Phosphatase hängt die Lage des p<sub>H</sub>-Optimums von der Substratkonzentration ab; ist diese niedrig, so ist die Kurve gegen neutrale p<sub>H</sub>-Werte hin verschoben. Wahrscheinlich ist die Substratkonzentration innerhalb der Zelle gering. Bei der Bestimmung der p<sub>H</sub>-Aktivitätskurven werden die Substratkonzentrationen im allgemeinen so gewählt, dass sie die Reaktionsgeschwindigkeit nicht beeinflussen. Diese Konzentrationen sind aber verhältnismässig gross, sodass sie keine Rückschlüsse auf das in der Zelle für die Aktivität der Phosphatase herrschende p<sub>H</sub>-Optimum erlauben.

3. Durch Dialyse konnte aus Nierenextrakten eine, die Wirkung der Phosphatase hemmende Substanz entfernt werden. Das p<sub>H</sub>-Optimum wurde durch die Dialyse nicht verändert.

4. Die Phosphomonoesterase I konnte an Kaolin adsorbiert und danach wieder eluiert werden. Das Enzym wurde dann mit Äthanol ausgefällt. Auf diese Weise gereinigte Präparate enthalten keine anderen Phosphomonoesterasen, wohl aber Pyrophosphatase I. Diese konnte durch Adsorption der Phosphomonoesterase an Diphenylmethanol bei saurem p<sub>H</sub> und Eluieren bei alkalischem p<sub>H</sub> entfernt werden.

5. Es wurde gezeigt, dass eine Anzahl von Kationen und Anionen auf die Lage des p<sub>H</sub>-Optimums der alkalischen Phosphomonoesterase keinen Einfluss haben.

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## A CRITICAL EXAMINATION OF THE HISTOCHEMICAL DEMONSTRATION OF THE ALKALINE PHOSPHOMONOESTERASE

by

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### INTRODUCTION

GOMORI<sup>1</sup> has—practically simultaneously with TAKAMATSU<sup>2</sup>—described a method to show the presence of phosphomonoesterase I—the so-called alkaline phosphatase—in tissue sections. The sections are placed in a solution containing a phosphoric acid ester and  $\text{Ca}^{++}$  ions. The phosphatase liberates  $\text{PO}_4^{--}$  ions from the ester, which with  $\text{Ca}^{++}$  ions yield the insoluble calcium phosphate. Hence the precipitate of calcium phosphate is formed in places where the enzyme happens to be present. The calcium phosphate is afterwards rendered visible by transformation into silver phosphate, which substance can be decomposed by light or by transforming it into cobalt phosphate, which can be converted into brown cobalt sulphide.

It is often recommended to let the ferment reaction take place at  $\text{pH} = 9.4$  (or still higher), as it is desirable to make use of the optimum  $\text{pH}$  of the phosphatase reaction. The solubility of the calcium phosphate that has been formed is also dependent on the  $\text{pH}$ . Finally GOMORI<sup>3</sup> recommends addition of  $\text{MgSO}_4$  as an activator.

The paraffin sections of objects fixed in alcohol or acetone are stretched on warm water in the usual way, placed on a slide and dried in a thermostate or (better) an exsiccator. After deparaffining in xylene, they come, after passing through ethanol (descending concentrations) and distilled water, into the substrate solution at  $37^\circ \text{C}$ .

In order to check this process some other sections are put in a solution containing  $\text{Ca}(\text{NO}_3)_2$  instead of substrate, for the rest they are treated analogously. In this manner insoluble Ca-compounds which have not been formed by the action of phosphatase are shown to be present or absent.

An investigation of the localization of the phosphomonoesterase I in the kidney showed that the pictures obtained by this method were not constant. We had the impression that with numerous nephrons the proximal part was incompletely stained and moreover in those parts in which the reaction could be called successful, the localization was mostly diffuse and blurred. The gradation of the blackening in various sections of the same object—also if these sections have at the same time been treated with the same substrate solution—varied considerably. We suppose that errors were already committed before the sections came into the substrate.

*References p. 135.*



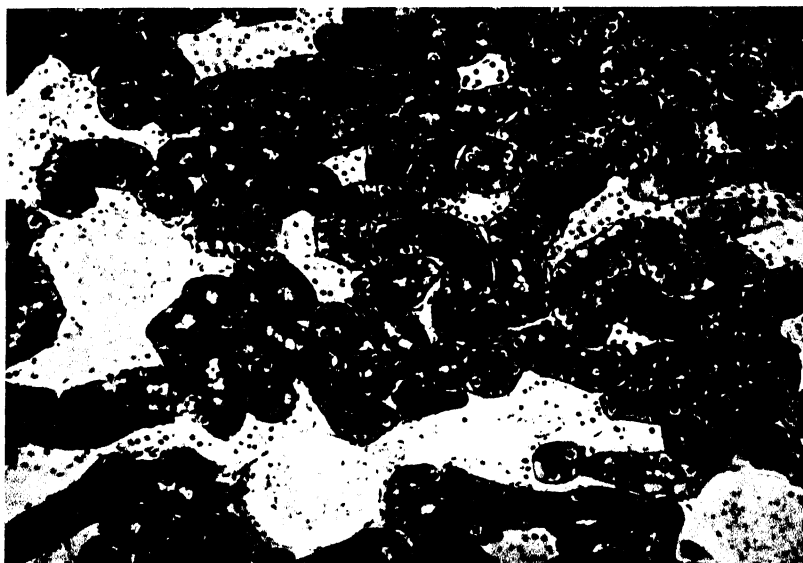


Fig. 1. Kidney of the guinea pig. Section of  $6\ \mu$  placed on a slide and transferred to the buffered substrate of  $pH = 9.4$  at  $32^\circ C$  for 20 hours

#### EXPERIMENTAL PART

##### 1. Fixation

The sections were fixed in alcohol of 80%. This proved to be the best fixative (see also DANIELLI<sup>4</sup>).

##### 2. Substrate solution

Most authors choose  $Na\text{-}\beta\text{-glycerophosphate}$  and  $Ca(NO_3)_2$  (GOMORI) for a substrate solution. The  $pH$  is adjusted with a buffer, if necessary.

##### 3. Enzyme preparation

To demonstrate that under these circumstances phosphomonoesterase I really reacts as described we carried out the following experiment. From extract of the kidney of a cow we made a purified preparation of alkaline phosphatase in a manner which has been described elsewhere<sup>5</sup>. We obtained the ferment as a white powder, which was washed out with ethanol 96% and subsequently suspended in ethanol. Now celloidin was added and after drying the whole was embedded in paraffin, of which "sections" were cut. As a matter of fact the blackening could be obtained in the way mentioned. A preparation in the control solution remained negative.

##### 4. Diffusion of the enzyme in the preparation

The following investigations were carried on with kidneys of the rat, mouse and guinea-pig. The kidney is an organ, the cortex of which, in contrast with the medulla, is rich in alkaline phosphatase, as biochemical research has proved<sup>6, 7</sup>. It has been described histochemically by a number of investigators, who particularly call attention to the localization of the ferment in the proximal part of the nephron.

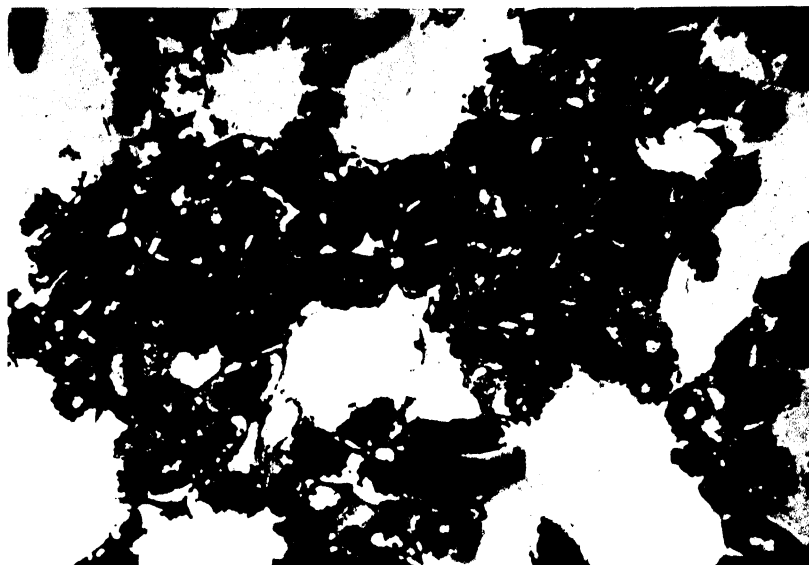


Fig. 2. Technique the same as in Fig. 1, but placed in ethanol 30% for half an hour before transferring to the substrate

Of a number of sections 6  $\mu$  thick part were mounted on slides freed of paraffin and again placed in water and part were transferred to the substrate solution at 32° C in a non-deparaffined condition. For these experiments we used an unbuffered solution of Na- $\beta$ -glycerophosphate and calcium nitrate (cf. GOMORI<sup>1</sup>). The non-deparaffined sections float on the surface of the warm fluid and stretch themselves. In principle this technique was derived from the data given by JACKSON<sup>8</sup>, who wanted to reattain the mutual cohesion of the structural parts in a tissue section during the action of trypsin.

After termination of the reaction we allowed the substrate solution to cool down to room temperature. The sections were washed in a calcium nitrate solution (0.5%) and then in distilled water. Subsequently the sections were placed in a 0.5% solution of silver nitrate and irradiated with u.v. light of a mercury lamp till the desired blackening was reached. During all these manipulations the sections float on the surface of the fluid. After termination of the histochemical reaction the sections were mounted on slides and dried. Removal of superfluous AgNO<sub>3</sub> and colouring, if any, of the sections took place with the deparaffined sections.

With this modified method we obtained much more constant pictures than with the deparaffined mounted sections treated in accordance with the original method. In addition to the proximal part of the nephron the cells of the basal layers of the pelvis epithelium also show a finely grained blackening.

A more or less identical result is obtained if the sections are deparaffined and mounted, but are then transferred directly from the xylene to the substrate solution, that is to say without letting them pass through the series of descending ethanol concentrations. As is well known the activity of the enzyme is retained after the treatment with ethanol. The transfer of the sections from ethanol 96% to water must be the cause of the varying pictures and atypical localization of the enzymically formed calcium phosphate. The chemical examination had shown that phosphomonoesterase

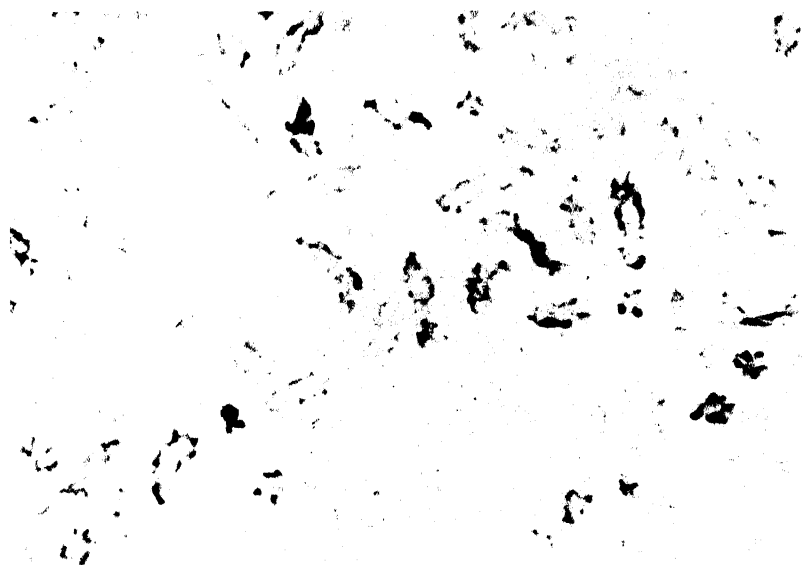


Fig. 3. Technique the same as in Fig. 1, but placed in ethanol 30 % for 24 hours before transferring to the substrate

I is readily soluble in dilute ethanol (30–50%); it is more soluble in this medium than in water. If a non-deparaffined section is treated with water for 20 hours this does not influence the reaction. With 30% ethanol the greater part of the ferment can be extracted in 20 hours. By interrupting the reaction after the lapse of different periods one can observe that the solution of the enzyme is accompanied by a diffusion of the latter to the surrounding parts of the tissue. *By taking sections from xylene to water via various ethanol concentrations the true picture of the localization of the enzyme is disturbed.*

##### 5. Control preparations. Inactivation of the ferment

A further control was now the inactivation of the phosphatase. If the blackening is indeed exclusively caused by enzyme activity, sections in which this enzyme has been destroyed must be perfectly negative.

a) *Destruction by heat.* Sections are immersed in boiling distilled water for one minute. After this treatment they are perfectly negative. On shorter heating the activity appears to have strongly decreased.

It was also possible to destroy the phosphatase by heating the sections or thin fragments of tissue of material embedded in paraffin, these being kept dry (temperature about 80° C).

b) *Destruction by irradiation.* We already know from the chemical examination of organ extracts that by irradiation with u.v.light the enzyme is destroyed and loses its activity. If sections (non-deparaffined) are irradiated for 15 minutes with a mercury lamp at a distance of 50 cm no more blackening is to be seen.

##### 6. Control preparations. Inhibition of the reaction

Finally we made the following control experiments:

The ferment reaction is inhibited with KCN-solution. Preparations that had been



Fig. 4. Non-deparaffined section transferred to the buffered substrate

immersed in a substrate solution containing traces of KCN were practically negative.

7. *Control preparations. Suppression of the substrate. The  $p_H$  of the reaction*

It is obvious that the enzyme reaction should occur at optimum  $p_H$ . Accordingly GOMORI<sup>9</sup> recommends a  $p_H = 9.4$ . Later authors sometimes choose a still higher  $p_H$ . The optimum  $p_H$  of the alkaline phosphatase of the kidney is (with a high substrate concentration) 9.8. As a matter of fact a number of sections that had reacted at various  $p_H$  showed that the blackening at optimum  $p_H$  was more intense.

At  $p_H = 9.4$  or higher in the non-deparaffined section not only blackening of the proximal part of the nephron had occurred but also of the other tubules both in the cortex and the medulla, of the glomeruli and also of part of the intertubular connective tissue.

The blackening was most intense in the proximal part, while that of the distal part of the nephron and that of the collecting tubules consisted of a thin and finely grained precipitate. It was typical of this reaction that the nuclei were strongly positive.

The picture is different with the mounted deparaffined sections, where the time of the substrate is much reduced. Now the blackening is chiefly localized in the proximal of the nephron: the cells contain a finely grained precipitate, which was diffuse and condensed especially in the neighbourhood of the cuticula and to a lesser degree of the basal part of the cell. In this preparation the nuclei in the cortical region were also positive. The latter not only applies to the epithelial cells, but also to those of the glomeruli and the intertubular cells of the connective tissue and to the epithelium of the pelvis. In the pelvis of the guinea pig only the basal layers of cells show a positive reaction. This bears out the observations of GOMORI<sup>9</sup>.

For control purposes sections were placed into a  $\text{Ca}(\text{NO}_3)_2$  solution (0.4%), which was brought to the desired  $p_H$  by means of a buffer. For the rest these control sections



Fig. 5. Non-deparaffined section transferred to a buffered  $\text{Ca}(\text{NO}_3)_2$  solution of  $\text{pH} = 9.4$  at  $32^\circ \text{C}$  for 20 hours

were treated in perfectly the same way as the sections that came into the substrate. Now non-deparaffined control sections were negative at a  $\text{pH} = 8.4$ , but at a  $\text{pH} = 9.4$  pictures were obtained here which were practically identical with the sections that had been treated with substrate at  $\text{pH} = 9.4$ . Only the blackening was less intense.

From these experiments it may be inferred that at  $\text{pH} = 9.4$  precipitates of insoluble Ca-compounds are formed, of which it is not known for certain that they are due to phosphatase action. The pictures obtained at  $\text{pH} = 9.4$  do not give an accurate idea of the localization of the alkaline phosphatase.

By washing the sections beforehand in distilled water for 24 hours these disturbing substances could be eliminated. The sections were then only positive in the proximal part of the nephron.

On introducing sections in which the ferment had been destroyed by dry heating in a  $\text{Ca}(\text{NO}_3)_2$  solution at  $\text{pH} = 9.4$  we found a blackening which was perfectly identical with that of the untreated sections. The same picture was obtained by placing the sections in the substrate solution of GOMORI at  $\text{pH} = 9.4$ . The enzyme proved to have become inactive as the typical picture of the localization of the enzyme in the proximal part of the nephron is entirely absent. From this it is evident that the blackening which is formed in the non-deparaffined sections that had been in the GOMORI-solution at  $\text{pH} = 9.4$  is not entirely due to phosphatase activity.

#### 8. *Some slight modifications*

As a substrate solution we have substituted Ca-glycerophosphate 0.4% for the mixture of Na- $\beta$ -glycerophosphate and calcium nitrate. The preparation of the former is simpler and moreover there are no superfluous ions in the solutions.

For rendering visible the Ca-phosphate that had been formed we preferred the silver method to the cobalt method, because sections, both deparaffined and non-



Fig. 6. Non-deparaffined section kept for 20 hours in distilled water at  $32^{\circ}\text{C}$ ; subsequently transferred to the buffered substrate

deparaffined, which had been in a  $\text{CoCl}_2$ -solution for some minutes (without any previous treatment) and had afterwards been in an ammonium sulphide solution, turned out not be negative. The nuclei in particular adsorbed Co. LISON<sup>10</sup> also points to this and to the fact that ammonium sulphide transforms many metals (e.g., Fe) into coloured sulphides.

As the conversion of  $\text{CaHPO}_4$  into  $\text{Ag}_3\text{PO}_4$  with  $\text{AgNO}_3$  is not entirely quantitative, owing to the  $\text{H}^+$ -ions that are formed, we tried to transform the  $\text{CaHPO}_4$  into  $\text{Ag}_3\text{PO}_4$  with a saturated solution of silver acetate in 1 m sodium-acetate. The acetate ions present in this solution ensure a quantitative progress of the reaction by their buffering action. The blackening obtained in this way turned out to be more sharply localized as compared to the method that makes use of  $\text{AgNO}_3$ .

#### CONCLUSION FROM THESE DATA

As a substrate solution a 0.4% solution of Ca-glycerophosphate is very satisfactory.

Deparaffined sections which have been transferred from xylene to water via various ethanol concentrations show a diffusion of the alkaline phosphatase and give blurred pictures.

The localization of the blackening in non-deparaffined sections is very sharp.

Non-deparaffined sections already give a positive reaction at  $\text{pH} = 9.4$  on being placed in a  $\text{Ca}(\text{NO}_3)_2$  solution (control sections). The nuclei are also black then.

At  $\text{pH} = 8.4$  the control sections are negative. Most nuclei in sections that have been in a substrate solution are negative at  $\text{pH} = 8.4$ .

Although in the literature it is often stated that many nuclei contain phosphatase, we must doubt if this may be concluded from the histochemical examination.

When the reaction takes place at  $\text{pH} = 9.4$  most nuclei show blackening. By

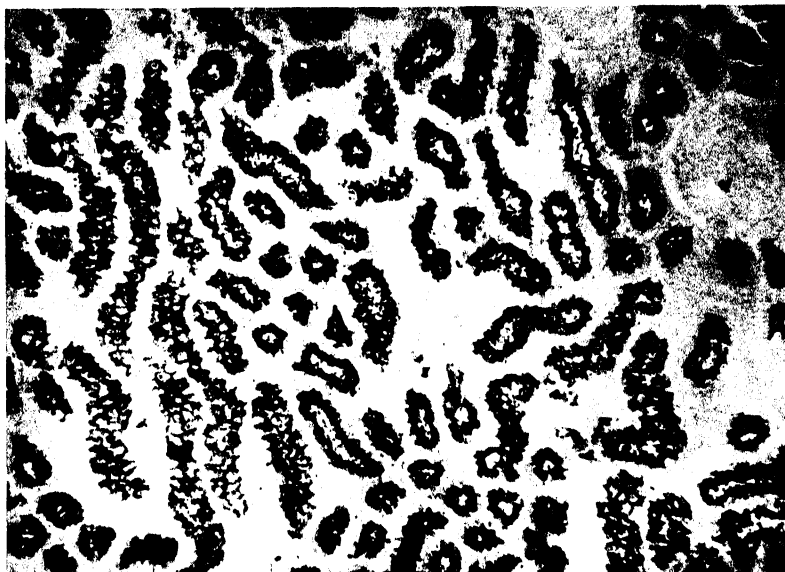


Fig. 7. Non-deparaffined section transferred to a 0.4 % solution of Ca-glycerophosphate of  $p_H = 8.4$  at  $32^\circ C$  for 20 hours

treating the sections with distilled water before the reaction a quite different picture is obtained. Now those nuclei are chiefly black which are in cells the cytoplasm of which shows great phosphatase activity. This picture shows great resemblance to that which is obtained by carrying out the reaction at  $p_H = 8.4$ .

LISON<sup>10</sup> points to the possibility that the lyo-enzymes may be easily washed out, in contrast to the desmo-enzymes. It might be that the nuclei mainly contained lyo-phosphatase which has been eliminated by the treatment with distilled water. If, however, the phosphatase has been destroyed by dry heating a blackening of the nuclei is still obtained at  $p_H = 9.4$  with and without a substrate. We have already observed that with the cobalt method a blackening of the nuclei arises already through adsorption of Co.

If instead of Na- $\beta$ -glycerophosphate +  $Ca(NO_3)_2$  a solution is used containing Na- $\beta$ -glycerophosphate +  $MgSO_4$  and if a  $NH_4Cl-NH_4OH$  buffer is used, then it is not calcium phosphate which precipitates but  $Mg(NH_4)PO_4$ , which may likewise be converted into  $Ag_3PO_4$ . With this substrate we can work at  $p_H = 9.4$  and even at  $p_H = 10.0$ , as the sections with  $MgSO_4$  in the  $NH_4Cl-NH_4OH$  buffer showed no blackening after washing and treatment with  $AgNO_3$  (control experiment). With this method most nuclei were negative.

We may observe that JUNGE, MENTEN, AND GREEN<sup>11</sup> and later DANIELLI<sup>4</sup>, using  $\beta$ -naphthol phosphate, phenyl phosphate or phenolphthaleine phosphate as substrate see no positive reactions of the nuclei. The rest of the picture was identical with that which was obtained with Na- $\beta$ -glycerophosphate as a substrate. LORCH<sup>12, 13</sup>, however, thinks that this is due to the above-mentioned reactions being less sensitive.

We believe that one must be very careful when interpreting the pictures. From a blackening of the nuclei it may not be inferred that these contain alkaline phosphatase. An investigation in which amongst other things different substrates are made use of is called for.

*References p. 135.*

## METHOD

We subjoin a description of the method now used by us to demonstrate the presence of alkaline phosphatase in tissue sections.

This fragments of an organ (2–3 mm) are fixed in ethanol 80% for 12–24 hours. The bottom of the vessel is covered with a thin layer of cotton-wool. The 80% ethanol is renewed once during this period. Subsequently the fragments are placed in 2 portions of 96% ethanol. Then in 2 portions of methyl benzoate for 12–24 hours. The fragments are transferred to xylene or toluene for 2–3 hours. From this they pass into 3 portions of molten paraffin (melting-point 54–56° C), for 30–45 minutes in each, and are embedded then.

As during manipulations with loose paraffin sections the organic connection of the tissue constituents generally does not remain intact in sections of this kind, especially during the stay in the heated substrate or control solutions, it is desirable to embed the fixed material in celloidin-paraffin.

When this method of embedding is applied absolute ethanol is substituted for methyl benzoate. From this the fragments pass into a 2% celloidin-solution (equal parts of absolute ethanol and acetone) for 24 hours. Subsequently the superfluous celloidin is allowed to drip off and the fragments are embedded in paraffin via xylene or toluene.

Now sections 6  $\mu$  thick are cut and directly placed into a 0.4% solution of Ca-glycerophosphate of  $p_H = 8.4$  at 32° C\*. This solution is prepared just before use. The sections must float on the surface of the fluid and they stretch themselves through the heat; air-bubbles or creases are removed with a thin glass rod or preparing needle. In this solution the sections remain for 20 hours; thereupon they are allowed to cool down to room temperature and are transferred to distilled water one by one with the aid of a little rod. Afterwards they are washed once more in distilled water and then transferred to a 0.5%  $AgNO_3$ -solution or a saturated solution of silver acetate in 1 m sodium acetate. This last solution is obtained by dropping a solution of  $AgNO_3$  (e.g., 0.5 gramme in 1 ml of water) into 1 m sodium acetate solution until a white precipitate settles out. The fluid is now filtered. When the sections are transferred to one of these fluids they must not be immersed. They remain in this fluid till a distinct blackening appears: in diffuse daylight this will happen after a few hours, the time being much shorter in case of irradiation by a mercury lamp. The latter method, however, has the drawback that there is a possibility of the silver reagent being partly decomposed.

Now the sections are placed on slides after being washed in distilled water, mounted with dilute albumen-glycerine, and dried for some hours in a thermostate at 32° C. The paraffin is now removed by means of xylene and the sections are transferred to distilled water via ethanol. They are washed for 5 seconds in a 1%  $Na_2S_2O_3$ -solution. After careful washing they can be stained, if this is desired (e.g., haematoxyline, if required in combination with eosin) and embedded in Canada balsam in the usual way.

One of the authors (N) owes many thanks to the ROCKEFELLER FOUNDATION which made it possible to continue this research.

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\* If the experiment is to be carried out at  $p_H = 9.4$  it is absolutely necessary to place the sections in distilled water at 32° C for a few hours beforehand.



## SUMMARY

1. A method has been described to localize the alkaline phosphomonoesterase in paraffin sections. The original method of GOMORI was used with various modifications however.

2. It is shown that a diffuse picture is obtained when the sections placed on a slide are brought into water via ethanol. This picture is formed by the solubility of the enzyme in diluted ethanol. It is recommended to execute this reaction with non-deparaffined sections.

3. It appears from control experiments where the substrate has been replaced by  $\text{Ca}(\text{NO}_3)_2$  that at  $\text{pH} = 9.4$  and higher a sediment of insoluble Ca-compounds appears in the sections which have not been formed by the action of phosphatase. This formation has not been obtained at  $\text{pH} = 8.4$ . If one would like to execute this reaction at  $\text{pH} = 9.4$  then it is necessary to wash out the sections with distilled water first.

4. If the reaction proceeds at  $\text{pH} = 8.4$  no blackening is to be perceived in sections in which the enzyme has been destroyed before the reaction either by heating or by u.v. light. This proves that the blackening which otherwise appears at  $\text{pH} = 8.4$  must in fact be ascribed to the action of the phosphatase.

At  $\text{pH} = 9.4$  a blackening has been formed in sections that have previously been heated with or without substrate. It is identical with that in the control sections (mentioned under 3).

5. From control experiments it has appeared that in the sections the blackening of the nuclei is not due to the phosphatase activity. In an extensive discussion it is pointed out that we may not conclude from the histochemical research that nuclei contain alkaline phosphatase.

## RÉSUMÉ

1. Description d'une méthode permettant de localiser la phosphomonoestérase alcaline dans des coupes à la paraffine. Il s'agit de modifications de la méthode originale de GOMORI.

2. Il se produit une image diffuse lorsque les coupes sont placées sur une lame dans l'eau, après avoir été traitées par l'alcool. Ce caractère diffus est dû à la solubilité de l'enzyme dans l'éthanol dilué. Il est recommandé de faire cette réaction avec des coupes non déparaffinées.

3. Des expériences de contrôle montrent que si l'on remplace le substrat par  $\text{Ca}(\text{NO}_3)_2$ , il se forme dans les coupes à  $\text{pH} 9.4$  et aux  $\text{pH}$  supérieurs, un dépôt de composés calciques insolubles qui ne correspondent à aucune action de la phosphatase. Cette formation n'a pas lieu à  $\text{pH} 8.4$ . Lorsque l'on veut exécuter la réaction à  $\text{pH} 9.4$ , il est donc indispensable de laver préalablement les coupes à l'eau distillée.

4. Lorsque l'essai a lieu à  $\text{pH} 8.4$ , il n'apparaît aucun noircissement dans les coupes où l'enzyme a été préalablement détruite, soit par chauffage, soit par lumière ultra-violette. Ceci montre que le noircissement qui apparaît à  $\text{pH} 8.4$  dans les conditions normales, est dû à l'action de la phosphatase. A  $\text{pH} 9.4$ , il apparaît un noircissement dans les coupes qui ont été préalablement chauffées, et ceci en présence ou en l'absence de substrat. Ce noircissement est identique à celui que l'on observe dans les coupes témoins (voir 3).

5. Des expériences de contrôle ont montré que le noircissement des noyaux dans les coupes n'est pas dû à l'activité de la phosphatase. Une discussion approfondie montre que les recherches histochimiques ne permettent pas de conclure, jusqu'à présent que les noyaux contiennent de la phosphatase alcaline.

## ZUSAMMENFASSUNG

1. Es wird eine Arbeitsweise beschrieben, welche die alkalische Phosphomonoesterase in Paraffinschnitten zu lokalisieren erlaubt. Dies ist eine Modifikation der Originalvorschrift von GOMORI.

2. Wenn die Schnitte, nach Behandlung mit Äthanol, in Wasser auf den Objektträger gebracht werden, entsteht ein verschwommenes Bild. Dies ist durch die Löslichkeit des Enzyms in verdünntem Äthanol bedingt. Es wird daher empfohlen, die Schnitte für diese Reaktion nicht von Paraffin zu befreien.

3. Ersetzt man das Substrat durch  $\text{Ca}(\text{NO}_3)_2$ , so entstehen in den Schnitten bei  $\text{pH} 9.4$  und bei höheren  $\text{pH}$ -Werten, Niederschläge von unlöslichen Calciumverbindungen, die nicht auf Phosphatasewirkung zurückzuführen sind. Diese Niederschläge entstehen nicht bei  $\text{pH} 8.4$ . Will man also die Reaktion bei  $\text{pH} 9.4$  ausführen, so muss man die Schnitte vorher mit destilliertem Wasser auswaschen.

4. Wird der Versuch bei  $\text{pH} 8.4$  ausgeführt, so entsteht keine Schwärzung in den Schnitten, in denen das Enzym vorher durch Erwärmen oder durch Ultraviolettstrahlen zerstört wurde. Dies beweist, dass die Schwärzung, die bei  $\text{pH} 8.4$  unter normalen Bedingungen auftritt, in der Tat auf Phosphatasewirkung zurückzuführen ist. Bei  $\text{pH} 9.4$  treten Schwärzungen in Schnitten auf, die vorher mit oder ohne Substrat erhitzt worden waren. Es sind dieselben Schwärzungen wie in den Kontrollversuchen (siehe 3).

5. Kontrollversuche haben gezeigt, dass in den Schnitten die Schwärzung der Kerne nicht auf Phosphataseaktivität zurückzuführen ist. Es wird ausführlich auseinandergesetzt, dass auf Grund von histochemischen Untersuchungen nicht auf das Vorkommen von alkalischer Phosphatase in den Zellkernen geschlossen werden kann.

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## BOOK REVIEW

*The Basis of Chemotherapy*, THOMAS S. AND ELIZABETH WORK. Oliver and Boyd Ltd., Edinburgh 1948, 435 pp. 26/- net.

This book gives magnificent demonstration of the change which has taken place in the spirit and content of chemotherapeutic research during the past 12 years. The emphasis must be on research and understanding, as is implied in the book's title. Practical achievements are reported, but, because the book so largely deals with the background of chemotherapeutic practice, the partial successes of the past are not superseded but are incorporated and reinterpreted; with ethyldihydrocuprein, still remembered as the first bacterial chemotherapeutic agent of 1910, came the first description of bacterial drug resistance. Therefore although the book begins with a valuable historical chapter, the results of 20-50 years ago are also assimilated and described with current findings and appear among the 800 odd detailed references.

The guiding principles of the book are those of the past 10 years research, as is indicated by the chapter headings: Cell Metabolism; Essential Metabolites; Enzyme Inhibition; Drug Antagonism; Drug Resistance and the Relation of Structure and Activity. One is half afraid that they bespeak a new orthodoxy. The text of the chapters is actually varied much beyond the subjects implied in the headings and no trend in thought or interpretation has been ignored. Some aspects of the subject which are most emphatically basic do however receive a rather scattered treatment, *e.g.*, an expounding of the chemotherapeutic system as one comprising host, parasite and drug; the interaction of host and parasite or of host and drug. It would be advantageous to collect these in the index, which is a good one, or in an early chapter. But in a subject with the complexities of chemotherapy, many systems can be devised for organizing the available data, and the various systems have complementary advantages. The virtue of that chosen by the authors is its suitability for expounding one of the main themes in the research work of the past 10 years. It is necessarily a new way of interpreting the subject and one worthy of trial on the scale of the present book.

Because the contact of chemotherapy with the biochemistry of its times is surprisingly new, cell metabolism receives a detailed explanation, and a good one. Much of this knowledge is common to all biochemistry and the authors' comment on the need for greater knowledge of the comparative biochemistry of different parasites and of host and parasite is a timely one. Many classical experiments as those of WARBURG on the Atmungsferment are worth retelling, and the account reaches to contemporary work. We can sympathize with the authors' closing quotation from HUME on *ad hoc* hypotheses. The book comes at a time when there is less excuse than ever in chemotherapy for such hypotheses and for explanations which are not open to experimental attack. The authors' attempt to assess the dependence of a cell on particular metabolic pathways by quantitative data on enzyme inhibition, concentration, and turnover number are especially noteworthy. Also, as the authors emphasize, much which was relegated to permeability differences has been found to be open to experimental observation.

H. MCLWAIN (London)

## ON THE DIFFUSION RATES OF BACTERIOPHAGES

by

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Measurements of diffusion constants have contributed much to our understanding of the nature of proteins and other high molecular substances in solution. It might be expected that diffusion studies of viruses would add to our knowledge of these substances, but not much work has been done in this field. As MARKHAM, SMITH, AND LEA<sup>1</sup> have pointed out, three methods have been applied to viruses. First, the techniques employed in the studies of proteins have been applied to several viruses. Since these techniques make use of optical methods for the determination of concentrations at various levels, a large amount of purified virus is required, so much that the method cannot at present be generally used. Second, the method of BOURDILLON<sup>2</sup> has also been tried, but the realization of the ideal conditions necessary for consistent results is apparently difficult. In the third place, the porous disc method of NORTHROP AND ANSON<sup>3</sup> has been used by several investigators to investigate plant viruses and bacteriophage. The method of NORTHROP AND ANSON has the advantage of being applicable to impure preparations in concentrations easily attained.

The porous disc method has been applied to bacteriophages by several workers<sup>4, 5, 6, 7, 8</sup> and values of the diffusion constants found have been much higher than would be expected from sedimentation studies. The studies on diffusion of bacteriophages have been most recently discussed by HERSHEY, KIMURA, AND BRONFENBRENNER<sup>8</sup> who have attempted to reconcile the size of T2K bacteriophage particles as seen in electron micrographs ( $80 \times 100 \text{ m}\mu$ ) with the size calculated from diffusion constants ( $4 \text{ m}\mu$ ). They believed that they had evidence of circulation of fluid through the porous disc and thus explained the high diffusion constants.

The multichambered analytical method of POLSON<sup>9, 10</sup> seems especially applicable to the study of diffusion constants of bacteriophages since with its use free diffusion occurs and possible anomalies arising from the presence of a porous disc do not occur. An additional advantage is that the cell need not be standardized with substances whose diffusion constants are known, such as KCl, since the values may be calculated directly. Preliminary results from applying the method to the T3 and T4 bacteriophages have already been published<sup>11</sup>.

In the present paper, more extensive results obtained with these bacteriophages are reported. The variation of diffusion constant with concentration described is pointed out.

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## APPARATUS

The cell used in these studies has already been described<sup>9, 10</sup>. A battery of six was used which was attached to a base plate provided with levelling screws. The cells may be more conveniently prepared as follows: a 1 cm hole is drilled through the center of a cylindrical piece of stainless steel, 5 cm in diameter and 7 cm long. Six cylindrical holes, 5 mm in diameter, are drilled midway between the center of the cylinder and the periphery to a depth of 5 mm from the other end. The cylinder is then cut into the following sections: (a) a thin section, 5 mm thick, to serve as cover; (b) a 1 cm section; (c) a 2 cm section; and (d) a basal section, 3.5 cm thick. The surfaces of the sections are carefully ground and polished so that in apposition they fit perfectly, forming six cylindrical cavities. They are held together with a center pin provided with a thumb screw. The pin is hollowed out to provide space for a thermometer. The sections of the cell are interchangeable.

A thin layer of silicone grease (DOW-CORNING) is applied to the surfaces of the individual sections to render them watertight when clamped together.

For use the cells are fixed in position on the base plate and alternate cavities filled with the solution to be studied and with suitable solvent. By rotation the solvent may be placed over the solution and after an appropriate time the sections may be isolated by rotating the upper sections to the cut-off position.

## CALCULATIONS

Diffusion constants were calculated from the following expression which has been derived elsewhere<sup>10</sup>.

$$D = \frac{S^2}{C_o^2 A^2} \cdot \frac{\pi}{t} = \frac{C^2 H^2}{C_o^2} \cdot \frac{\pi}{t} \quad (1)$$

where  $D$  = the diffusion constant of Fick's law,  $S$  = the amount of solute which has diffused past the initial boundary between solution and solvent in  $t$  sec,  $C_o$  = the original concentration of the solution,  $A$  = the area of cross section in  $\text{cm}^2$ ,  $C$  = the mean concentration of solute in the upper section of the cell at time  $t$ , and  $H$  = the height of the upper section in cm.

From equation (1) the diffusion constant can be calculated. To convert the diffusion constant at one temperature  $T_1$  into that at another temperature  $T_2$ , the following equation was employed:

$$D_{T_2} = D_{T_1} \frac{T_2}{T_1} \cdot \frac{\eta_{T_1}}{\eta_{T_2}} \quad (2)$$

where  $D_{T_1}$  and  $\eta_{T_1}$  are the diffusion constant and viscosity at the absolute temperature  $T_1$ , and  $D_{T_2}$  and  $\eta_{T_2}$  those at the absolute temperature  $T_2$  respectively.

The particle sizes were calculated from the diffusion constants using the well-known Einstein equation for a spherical particle.

$$D = \frac{RT}{N} \cdot \frac{1}{6\pi r\eta} \quad (3)$$

where  $R$  is the gas constant,  $T$  is the absolute temperature,  $N$  is Avogadro's number,  $r$  is the radius of the particle, and  $\eta$  is the viscosity of the medium.

#### METHODS

*Stabilizing the Diffusion Column.* As extremely dilute solutions (w/o) of virus were employed, it was necessary to stabilize the liquid column into which the process of diffusion took place, because there was too little density difference between the solution and solvent. For that reason, the virus solution was made up in glucose of 1% concentration. This made it possible to obtain a sharp initial boundary between virus solution and medium. In the resulting diffusion process, the glucose establishes a concentration gradient in the cavities in which the virus then diffuses, undisturbed by convection currents. This addition of glucose had little effect on the diffusion of a protein as will be shown later.

The phage concentrations were determined by the plaque counting method. It was attempted to count 50–300 plaques for each determination. The hemoglobin concentrations were determined with the use of the Beckmann spectrophotometer after conversion of hemoglobin to acid hematin. KCl was determined chemically.

*Test of the Method on Substances of Known Diffusion Constants.* As a control of the method the diffusion constant of KCl was determined. Unfortunately, a relatively high concentration of salt was necessary in order to obtain sufficient material for analysis. 2.95% KCl was diffused against water and a diffusion constant of  $1.18 \cdot 10^{-5}$  cm<sup>2</sup>/sec at 4° C was calculated. After corrections for temperature and viscosity this becomes  $1.02 \cdot 10^{-5}$  cm<sup>2</sup>/sec at 0° C which is about 12% higher than that reported for KCl by LONGSWORTH<sup>12</sup>.

Another control of the method was made with human CO hemoglobin. This protein in a concentration of 4.4% in the presence of 1% glucose and 0.15 M NaCl was diffused against 0.15 M NaCl in the same manner as was used below for phage. The relationship  $C/C_0$  for various times is plotted against  $\sqrt{t}$  in Fig. 1. As can be seen, the resulting curve is a straight line passing through the origin. The diffusion constant calculated from this experiment is  $4.2 \cdot 10^{-7}$  cm<sup>2</sup>/sec which when corrected for temperature and viscosity becomes  $6.70 \cdot 10^{-7}$  cm<sup>2</sup>/sec at 20° C. Considering the high protein concentration used in this experiment, the agreement between this value and that determined for human hemoglobin by the Lamm scale method,  $6.90 \cdot 10^{-7}$  cm<sup>2</sup>/sec<sup>13</sup> is satisfactory. The glucose that was used for providing a density gradient in the diffusion column had no apparent effect on the progress of diffusion of a protein apart from the usual viscosity retardation effect.

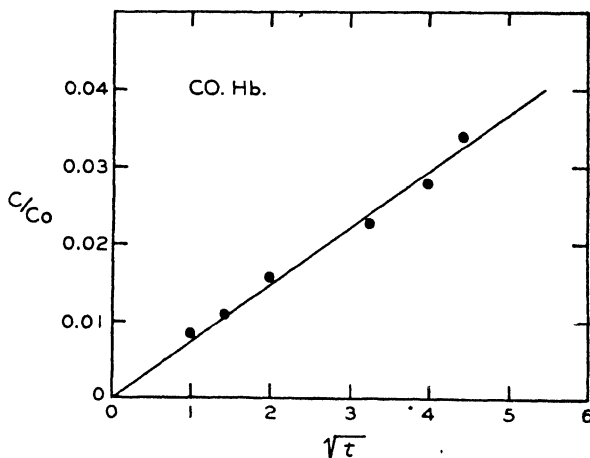


Fig. 1. Diffusion curve of carbon monoxide hemoglobin

## MEASUREMENTS

Unless otherwise stated, the phage solution to be diffused consisted of a dilution of phage in 9 parts of tryptose phosphate broth (Difco) plus 1 part of 10% glucose, and the solvent consisted of tryptose phosphate broth only. This medium has a  $p_H$  of

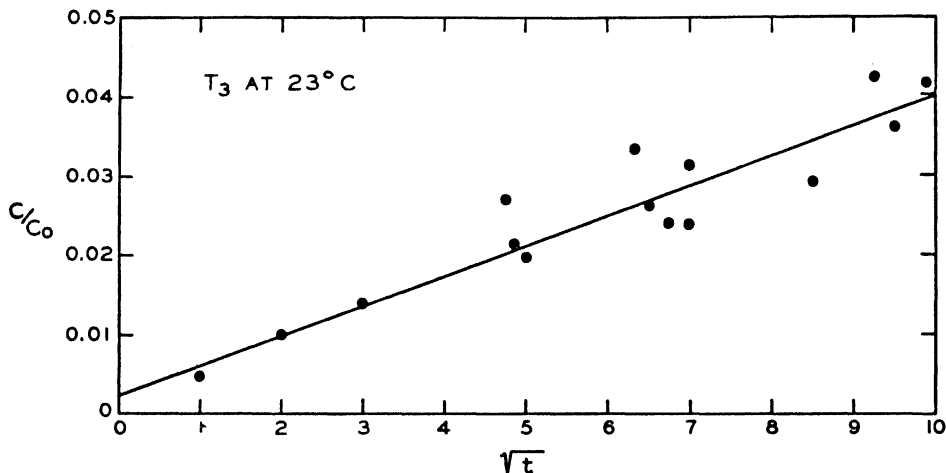


Fig. 2. Diffusion curve of  $T_3$  bacteriophage at  $23^\circ\text{C}$

about 7.3 and contains 0.5% NaCl and 0.25%  $\text{Na}_2\text{HPO}_4$ . In Fig. 2 are given results of measurements at  $23^\circ\text{C}$  on  $T_3$  phage solutions having phage contents of  $10^8$  and  $10^9$  particles/ml. The values are scattered around a straight line from which an average diffusion constant of  $1.19 \cdot 10^{-7} \text{cm}^2/\text{sec}$  at  $20^\circ\text{C}$  in water was calculated. From this value a particle diameter of  $36.2 \text{ m}\mu$  for  $T_3$  was calculated which is slightly smaller than the value reported by DELBRÜCK from measurements on electron micrographs<sup>14</sup>.

The same types of measurement were made on  $T_4$  phage having  $10^8$  and  $10^9$  particles/ml with the resultant curve in Fig. 3. Again the values determined for  $C/C_0$

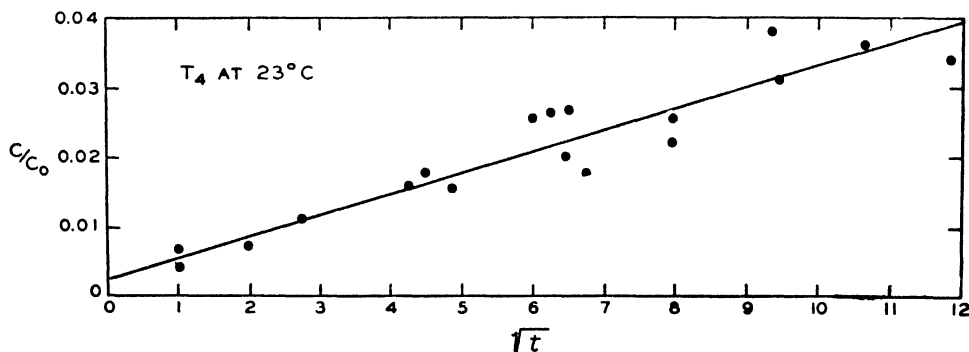


Fig. 3. Diffusion curve of  $T_4$  bacteriophage at  $23^\circ\text{C}$

are scattered around a straight line. From the straight line a diffusion constant of  $0.8 \cdot 10^{-7} \text{cm}^2/\text{sec}$  was obtained from which a particle diameter of  $55 \text{ m}\mu$  was calculated. This value is also in fair agreement with that reported by DELBRÜCK<sup>14</sup>.

As the determinations were scattered rather much around the mean, it was decided

to repeat them at 4° C at which temperature the most ideal diffusion constants can be determined on account of great stability of the phage and least probability of thermal convection currents.

The results did not come out as would be predicted from temperature and viscosity considerations alone, namely low rates of diffusion were slightly higher at this temperature than at 23° C. This can be seen from Figs 4 and 5.

The effect of concentration on diffusion constant was investigated. The results are interesting in that there was a very strong dependency of diffusion rate on the number of phage particles per ml. In Figs 6 and 7 and Table I are given the results at 4° C and at 22° C. At 4° C the diffusion values range from  $1.0 \cdot 10^{-7}$  cm<sup>2</sup>/sec to  $5.34 \cdot 10^{-7}$  cm<sup>2</sup>/sec uncorrected for temperature and viscosity for concentrations ranging from  $2 \cdot 10^{10}$  particles/ml to  $2 \cdot 10^5$  particles/ml.

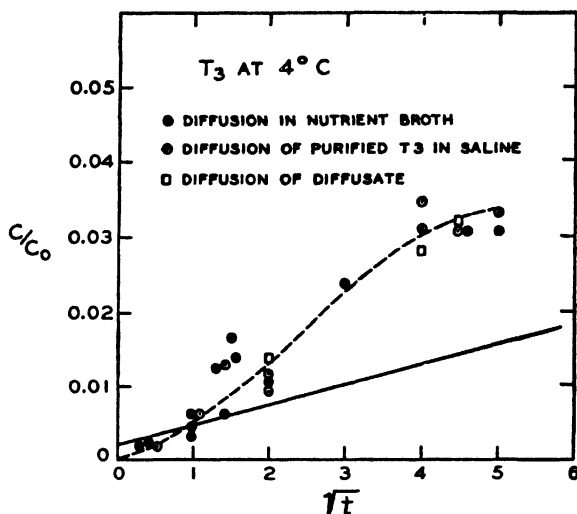


Fig. 4. Diffusion curve of T<sub>3</sub> bacteriophage at 4° C. The solid line expresses the value of  $C/C_0$  calculated from the results of Fig. 2, according to Equation (2).

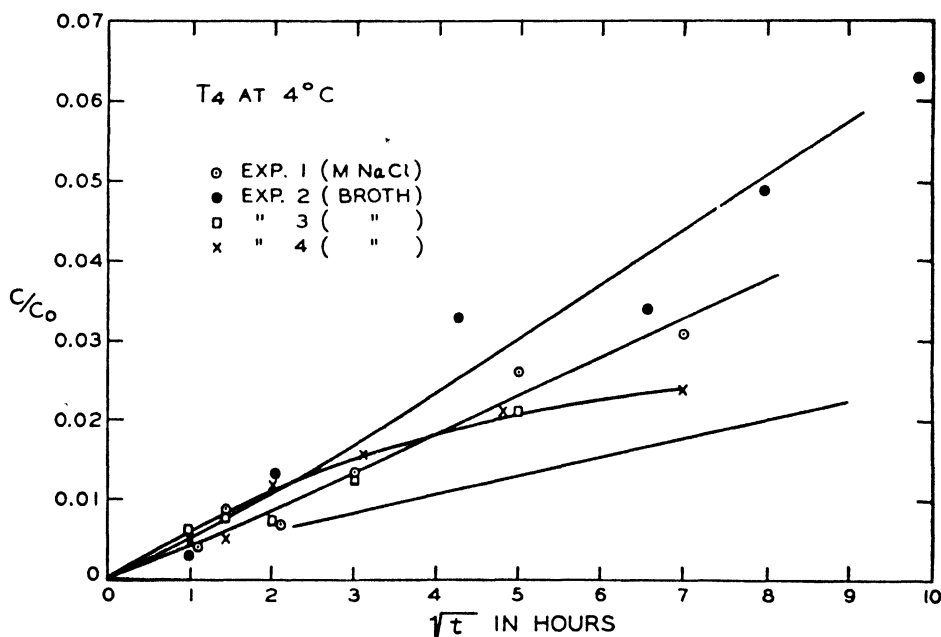


Fig. 5. Diffusion curve of T<sub>4</sub> bacteriophage at 4° C. The upper curves refer to different experiments at different original concentrations. The lowest curve expresses the values of  $C/C_0$  calculated from the results of Fig. 3, according to Equation (2).



At 22° C the variation was greater, here the diffusion constants ranged from  $3 \cdot 10^{-7} \text{cm}^2/\text{sec}$  to  $23.2 \cdot 10^{-7} \text{cm}^2/\text{sec}$  for the range of concentration  $25 \cdot 10^8$  to  $25 \cdot 10^4$  particles/ml.

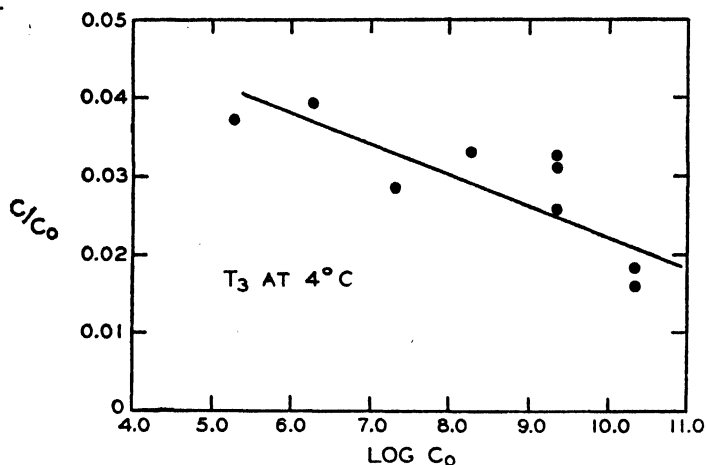


Fig. 6. A plot showing the variation of the ratio  $C/C_0$  with log of  $C_0$  for  $T_3$  bacteriophage at 4° C

Several experiments were conducted without success to find an explanation for this anomalous behaviour of the bacteriophage.

*Charge effects.* To exclude the possibility that the high diffusion rate was caused

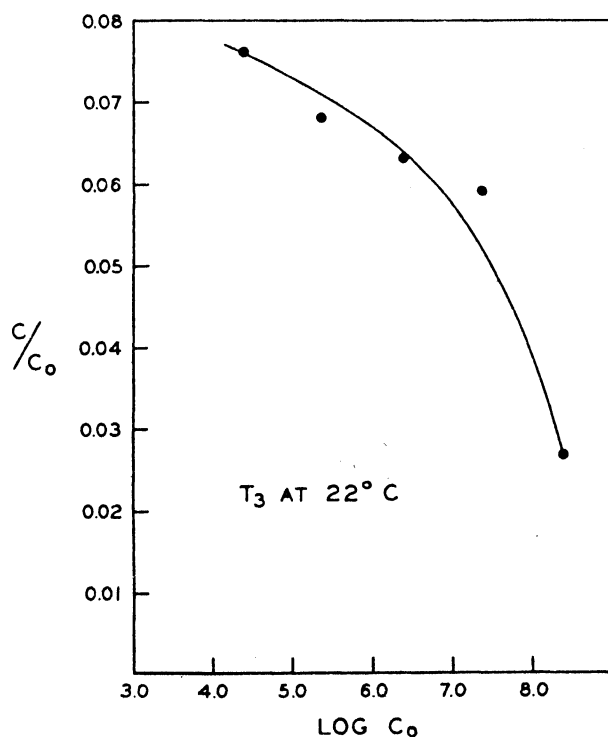


Fig. 7. Variation of the ratio  $C/C_0$  with log of the concentration for  $T_3$  bacteriophage at 22° C

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by charges on the phage particles, a diffusion experiment was made in the presence of 0.8 to 0.9 M NaCl. This high concentration of electrolyte did not change the diffusion rate of T4 significantly as can be seen in Fig. 5 and Table II.

TABLE I  
DIFFUSION OF T3 PHAGE FOR 20 HOURS AT 4°C AT VARIOUS CONCENTRATIONS

Dilution of Stock Solution	$C_0$	C	$C/C_0$
$10^5$	$197 \cdot 10^3$ *	$73 \cdot 10^2$	0.037
$10^4$	$174 \cdot 10^4$	$68 \cdot 10^3$	0.039
$10^3$	$195 \cdot 10^6$	$65 \cdot 10^6$	0.033
$10^1$	$230 \cdot 10^7$	$75 \cdot 10^6$	0.033
$10^0$	$223 \cdot 10^8$	$41 \cdot 10^7$	0.018
$10^3$	$224 \cdot 10^5$	$64 \cdot 10^4$	0.029
$10^1$	$282 \cdot 10^7$	$73 \cdot 10^6$	0.026
$10^1$	$247 \cdot 10^7$	$77 \cdot 10^6$	0.031
$10^0$	$218 \cdot 10^8$	$35 \cdot 10^7$	0.016

\*  $197 \cdot 10^3$  signifies the estimated number of phage particles per ml, and that 197 plaques were counted to make the estimate.

TABLE II  
DIFFUSION OF T1 PHAGE IN 0.9 M NaCl INTO 0.8 M NaCl

t (h)	$C_0$	C
1:15	$50 \cdot 10^6$	$193 \cdot 10^3$
2:00	$55 \cdot 10^6$	$49 \cdot 10^4$
4:20	$52 \cdot 10^6$	$36 \cdot 10^4$
9:00	$57 \cdot 10^6$	$75 \cdot 10^4$
25:00	$55 \cdot 10^6$	$143 \cdot 10^4$
49:00	$62 \cdot 10^6$	$191 \cdot 10^4$

TABLE III  
DIFFUSION OF T3 PHAGE PURIFIED BY ULTRACENTRIFUGATION. T = 4°C

t (h)	$C_0$	C	$C/C_0$	D
4:00	$40 \cdot 10^6$	$46 \cdot 10^4$	0.012	$3.7 \cdot 10^{-7}$ (Av.)
16:00	$35 \cdot 10^6$	$108 \cdot 10^4$	0.031	
20:00	$46 \cdot 10^6$	$142 \cdot 10^4$	0.031	

*Diffusion of Purified Virus.* It was thought that the high diffusion rate of the virus at low concentration might be altered by using ultracentrifugally purified phage. In this experiment, T3 phage in 200 ml solution, having a particle count of  $4 \cdot 10^{10}$  particles/ml, was sedimented twice at 15,000 rpm in the air-driven ultracentrifuge and the final, pellet dissolved in 2 ml saline; the particle count was now  $29 \cdot 10^{11}$ . 0.01 ml of this solution was diluted in a 0.02 M phosphate buffer of  $p_H$  7.4 containing 1.7% NaCl and diffused into 0.02 M phosphate buffer  $p_H$  7.4 containing 0.85% NaCl. Both solutions were filtered

for sterility. The particle count of the filtrate to be used for diffusion was now  $64 \cdot 10^7$  particles/ml. This solution was used for diffusion at  $4^\circ \text{C}$  into its buffer for various times. In Fig. 4 and Table III the results are given. As can be seen, the diffusion rate is still very high and the purification process had no detectable effect on it.

*Diffusion of Diffusate of T<sub>3</sub>.* In this experiment the diffusate of a previous diffusion experiment was diffused in broth in the usual way to investigate possible inhomogeneity. The resulting values of this experiment are presented in Fig. 4 and Table IV.

TABLE IV  
DIFFUSION OF DIFFUSATE OF T<sub>3</sub> PHAGE. T =  $4^\circ \text{C}$

t (h)	C <sub>0</sub>	C	C/C <sub>0</sub>	D
4:00	$27 \cdot 10^5$	$37 \cdot 10^3$	0.014	$3.8 \cdot 10^{-7}$ (Av.)
16:00	$35 \cdot 10^5$	$98 \cdot 10^3$	0.028	
20:00	$25 \cdot 10^5$	$79 \cdot 10^3$	0.032	

#### DISCUSSION

The results of NORTHROP<sup>6</sup>, when expressed in the units we have used, are of interest. In that work, concentrated solutions of a staphylococcus phage which contained  $10^{12}$  particles per ml or more gave small diffusion values ( $D = \text{about } 1 \cdot 10^{-8} \text{cm}^2/\text{sec}$ ). When the solution was diluted to contain about  $10^{11}$  to  $10^{10}$  particles per ml higher diffusion constants were obtained ( $D = \text{about } 2 \cdot 10^{-7} \text{cm}^2/\text{sec}$ ). The lower limit of the range studied by NORTHROP is about the same as our upper limit ( $10^{10}$  particles per ml), and the value observed by us ( $D = 1.0 \cdot 10^{-7} \text{cm}^2/\text{sec}$ ) is about half that seen by NORTHROP. At the lower limit of concentration studied by us, where the particle count was about  $10^5$  per ml, the diffusion constant was calculated to be  $5 \cdot 10^{-7} \text{cm}^2/\text{sec}$ . The diffusion experiments of NORTHROP were carried out at  $10^\circ$  and ours were at  $4^\circ$ . NORTHROP suggested that the variation in diffusion constant was evidence of dissociation of phage molecules as a result of dilution. In NORTHROP's investigation the activity method of KREUGER was used to measure phage concentrations.

Recently HERSHEY, KIMURA, AND BRONFENBRENNER<sup>8</sup> have suggested that the porous disc method for determining diffusion constants is unreliable because of circulation of fluid through the disc. They have reviewed their earlier work concerning heterogeneity in size of phage particles and regard the evidence for this heterogeneity in size as being insufficient.

The work here reported again shows the variation in diffusion constants with concentration. It does not appear to be evidence for heterogeneity since centrifugally purified phage and the diffusates of diffusion experiments gave the expected values for diffusion constants.

The preliminary work published by one of us, POLSON<sup>11</sup> was performed on phage solutions having particle counts of  $10^8$ – $10^{10}$  per ml. The diffusion constants were of the order of magnitude that would be expected from the sizes of bacteriophages as shown by electron micrographs. However, it can be seen from the results reported above, that the relatively low diffusion constants previously reported are explained by the dependency on concentration.

Alternatively it is suggested that these unexpectedly high diffusion constants

would result if the phage particles possess an independent specific motility in addition to the normal diffusion. This has not definitely been proved but additional evidence from ultrafiltration experiments has been obtained to support it.

### SUMMARY

The diffusion constants of T<sub>3</sub> and T<sub>4</sub> bacteriophages were studied by means of the multi-chamber analytical diffusion cell. The composition of the medium had no noticeable effect on the values found, but the concentration of the phage and temperature had pronounced effects on the corrected diffusion constants. At 4° C the diffusion constant of T<sub>3</sub> varied from  $5.3 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  to  $1.0 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  in the concentration range  $10^6$  to  $10^{10}$  particles/ml. At 22° C the diffusion constants varied from  $23 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  to the  $3 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  in the concentration range  $10^6$  to  $10^9$  particles/ml. These diffusion constants are much higher than would be expected from the size observed by electron microscopy.

### RÉSUMÉ

Les constantes de diffusion des bactériophages T<sub>3</sub> et T<sub>4</sub> ont été étudiées à l'aide de la cellule analytique de diffusion à plusieurs compartiments. La composition du milieu n'a pas d'effet notable sur les valeurs trouvées, mais la concentration des phages et la température ont des effets marquants sur la valeur des constantes de diffusion corrigées. A 4° C, la constante de diffusion de T<sub>3</sub> varie de  $5.3 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  à  $1.0 \cdot 10^{-7} \text{ cm}^2/\text{sec}$ , dans des zones de concentration allant de  $10^6$  à  $10^{10}$  particules/ml. A 22° C, les constantes de diffusion varient de  $23 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  à  $3 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  pour des zones de concentration allant de  $10^6$  à  $10^9$  particules/ml. Ces constantes de diffusion sont beaucoup plus élevées que celles auxquelles on pourrait s'attendre d'après la taille des particules observées au microscope électronique.

### ZUSAMMENFASSUNG

Die Diffusionskonstanten von T<sub>3</sub> und T<sub>4</sub> Bakteriophagen wurden mit Hilfe der analytischen "Multi-chamber" Diffusionszelle untersucht. Die Zusammensetzung des Mediums hatte keinen merkbaren Effekt auf die erhaltenen Werte, aber die Phagenkonzentration und die Temperatur hatten ausgesprochene Wirkungen auf die korrigierten Diffusionskonstanten. Bei 4° C variierte die Diffusionskonstante von T<sub>3</sub> von  $5.3 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  bis zu  $1.0 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  im Konzentrationsbereich  $10^6$  bis  $10^{10}$  Teilchen/ml. Bei 22° variierten die Diffusionskonstanten von  $23 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  bis zu  $3 \cdot 10^{-7} \text{ cm}^2/\text{sec}$  im Konzentrationsbereich  $10^6$  bis  $10^9$  Teilchen/ml. Diese Diffusionskonstanten sind viel höher als die im Elektronenmikroskop wahrgenommene Grösse erwarten liesse.

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CALCUL DES FRANGES DE DIFFRACTION OBSERVÉES  
SUR LES CLICHÉS D'ÉLECTROPHORÈSE OBTENUS  
PAR L'APPAREILLAGE DE LONGSWORTH

par

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Les phénomènes de diffraction que l'on peut observer sur les clichés photographiques obtenus au moyen des appareils d'électrophorèse du type LONGSWORTH-MAC INNES, ont été décrits par LONGSWORTH<sup>1</sup>.

La disposition des franges et la présence du couteau sont déjà, à première vue, une indication que le phénomène rentre dans le cas général de la diffraction de la lumière par un écran à bord droit<sup>2</sup>. En réalité, il s'agit d'un cas particulier plus compliqué, car l'écartement des franges varie avec le gradient: elles sont d'autant plus fines et serrées que le gradient est élevé et que sa variation est forte. Néanmoins, l'analogie est suffisante pour que l'on puisse déjà prévoir, comme le fait implicitement LONGSWORTH, que l'ombre géométrique — qui correspond au contour exact des courbes de gradient — est définie, sur les clichés, par le lieu des points où l'intensité lumineuse ( $I$ ) est égale au quart de l'intensité ( $I_0$ ) reçue sur la plaque en un point où le couteau ne produit pas d'ombre.

On sait que le repérage du contour exact est très important. La surface délimitée par la courbe de gradient et la ligne de base est proportionnelle à la concentration de la protéine responsable de la limite. La forme de la courbe doit être connue avec précision pour les mesures de diffusion que l'on peut faire avec le même dispositif optique.

On peut faire coïncider le lieu des points d'intensité  $\frac{I_0}{4}$  avec la limite ombre-lumière observée sur la plaque, en choisissant soigneusement les temps de pose et de développement et le matériel photographique. Le repérage du contour se fait alors facilement soit par photométrie, soit par calques et agrandissements. La luminosité de l'appareil dépendant de la nature des extraits protéiniques examinés, il n'est pas toujours facile de réaliser ces conditions de technique photographique et la précision du repérage peut en souffrir. Les franges de diffraction, par contre, sont aisément repérables et il serait possible, si l'on désire une très grande précision (dans le cas de mesures de diffusion par exemple), de construire par points la courbe à partir du lieu d'une frange nette, à condition de connaître l'écartement des franges pour chaque valeur du gradient. La connaissance de ces écartements permettrait aussi de vérifier si la technique photographique est au point. Ces quelques considérations et aussi l'intérêt théorique du phéno-

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mène nous ont conduit à calculer, en utilisant les procédés courants de l'optique physique, la position des franges de diffraction et à vérifier qu'il s'agissait bien d'un cas particulier de la diffraction par un bord droit.

### A. THÉORIE

Les divers éléments essentiels d'un appareil du type LONGSWORTH sont représentés sur la Fig. 1. La cellule est éclairée par un faisceau de lumière parallèle monochromatique. La source S est une fente horizontale très étroite. Le plus souvent, on utilise une seule grande lentille (Schlierenlinse) placée devant la cellule qui est alors éclairée par un faisceau convergent. Les calculs sont plus simples en lumière parallèle et les résultats seront valables pour un système convergent si la convergence est faible, cas toujours réalisé.

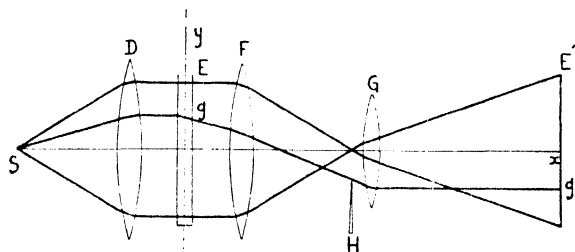


Fig. 1. Schéma du dispositif optique de l'appareillage électrophorétique du type LONGSWORTH

Rappelons que l'image E' est limitée par une fente verticale très étroite, placée devant la plaque photographique. Le plan x y (plan de la feuille) pourra donc nous servir à définir complètement les différents plans et les surfaces d'ondes que nous aurons à considérer. L'axe y définit le plan objet dans la cellule.

La lumière provenant de la cellule est diffractée sur le plan E' par l'ouverture délimitée par le bord droit du couteau H et les bords de la lentille G.

L'évaluation de l'intensité lumineuse sur l'image E' peut se faire en remplaçant la source lumineuse réelle par des sources fictives convenablement réparties sur la surface d'onde délimitée par l'ouverture considérée (principe de Huygens). Chaque point de cette source éclaire le plan E' et on trouvera l'intensité lumineuse en un point de l'image en faisant la sommation de l'action des sources fictives sur ce point, compte tenu des différences de phases.

Dans le cas qui nous occupe, le problème est compliqué par la présence de lentilles qui interviennent dans l'évaluation des chemins optiques et parce que la répartition de l'intensité sur la source de Huygens n'est pas uniforme à cause des gradients d'indice dans la cellule.

La difficulté due aux lentilles peut être résolue facilement de la façon suivante. Formons l'image de E' par rapport à l'ensemble F, G; cette nouvelle image E'' est confondue avec le plan y z dans la cellule. Formons également l'image du couteau, que nous supposons en  $y = 0$  (voir plus loin), par rapport à la lentille F; cette image sera rejetée à l'infini, à gauche ou à droite de la cellule. Tout se passe donc comme si la cellule E envoyait de la lumière sur un demi plan à l'infini. La trajectoire des rayons s'obtient en prolongeant les rayons réels, définissant ainsi la répartition à l'infini des sources de Huygens, qui diffractent de la lumière sur chaque point de l'image E'' confondue avec la cellule E.

La difficulté due à la répartition non uniforme de l'intensité lumineuse sur la source de Huygens peut être résolue par un changement de variable qui consiste à définir la position des rayons dans le système x, y de la cellule où l'éclairement est constant. La

sommentation des actions lumineuses dues aux sources fictives se fera tout le long du faisceau non intercepté par le couteau.

Il ne nous reste plus qu'à définir la surface d'onde due au gradient dans la cellule. Nous admettrons, pour simplifier, que le gradient d'indice dans la cellule est linéaire et de la forme:

$$\frac{dn}{dy} = -2a y \quad (a \text{ étant une constante positive}) \quad (1)$$

D'où on tire:

$$n = n_0 - a y^2 \quad (2)$$

La surface d'onde après passage dans la cellule est définie par:

$$e n + x = e n_0 + E \quad (3)$$

$e$  est le chemin parcouru dans la cellule, soit l'épaisseur de la cellule, les déviations étant petites;  $x$  est le chemin dans l'air après le passage de l'onde dans la cellule;  $E$  est une constante.

De (2) et (3), on tire:

$$x = e(n_0 - n) + E = e a y^2 + E \quad (4)$$

La vibration élémentaire envoyée par un élément de l'onde  $\Omega$  (Fig. 2) dans la cellule, sur la source de HUYGENS à l'infini, est de la forme

$$c' \cos\left(\omega t - \frac{2\pi r}{\lambda}\right) \text{ où } r \text{ est la distance séparant la cellule de la source}$$

diffRACTANTE. Les autres symboles ont leur signification habituelle. La vibration élémentaire diffractée en un point  $y_1$  de l'image  $E''$  sera, en ramenant le tout aux coordonnées de la cellule et en prenant a suffisamment petit:

$$dS = C dy \cos\left(\omega t + \frac{2\pi \delta}{\lambda}\right) \quad (5)$$

$\delta$  est la différence de marche entre le rayon issu d'un point d'ordonnée  $y$  dans la cellule  $E$  et le rayon diffracté en  $y_1$  sur l'image  $E''$ .

On a (Fig. 2):

$$\delta = -a(y_1 - y) \quad a \text{ étant petit} \quad (6)$$

La relation (4) nous donne:

$$a = \frac{dx}{dy} = 2e a y \quad (7)$$

On trouve, en combinant (6) et (7):

$$\delta = -2e a (y_1 - y) y \quad (8)$$

Choisissons un cas particulier et admettons qu'en  $y = 0$  il y ait un gradient tel que, pour masquer le pinceau lumineux dévié correspondant, le bord du couteau doive être placé en  $y = 0$  également.

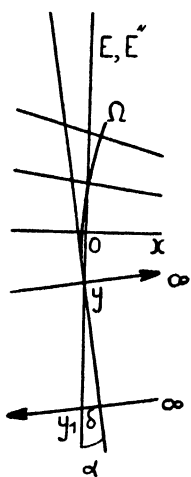


Fig. 2

Dans ces conditions, l'action totale de la source de HUYGENS sur le point  $y_1$  sera,  $h$  étant l'ordonnée du bord extrême de la cellule :

$$S = C \int_{-h}^0 \cos \left[ \omega t - \frac{4\pi e a}{\lambda} (y_1 - y) y \right] dy \quad (9)$$

ou en développant :

$$S = C \cos \omega t \int_{-h}^0 \cos A (y_1 - y) y dy + C \sin \omega t \int_{-h}^0 \sin A (y_1 - y) y dy \quad (10)$$

On voit que  $S$  est de la forme  $E \cos \omega t + F \sin \omega t$  et l'intensité au point  $y_1$  sera donnée par :

$$I = C^2 (E^2 + F^2) \quad (11)$$

On peut écrire successivement\* :

$$\begin{aligned} E &= \int_{-h}^0 \cos A (y_1 - y) y dy = \int_{-h}^0 \cos \left[ A \left( y - \frac{y_1}{2} \right)^2 - \frac{A y_1^2}{4} \right] dy \\ &= \cos \frac{A y_1^2}{4} \int_{-(h + \frac{y_1}{2})}^{-\frac{y_1}{2}} \cos A u^2 du + \sin \frac{A y_1^2}{4} \int_{-(h + \frac{y_1}{2})}^{-\frac{y_1}{2}} \sin A u^2 du \end{aligned} \quad (12)$$

$$F = \cos \frac{A y_1^2}{4} \int_{-(h + \frac{y_1}{2})}^{-\frac{y_1}{2}} \sin A u^2 du - \sin \frac{A y_1^2}{4} \int_{-(h + \frac{y_1}{2})}^{-\frac{y_1}{2}} \cos A u^2 du \quad (13)$$

En posant  $\int \sin A u^2 du = \theta$  et  $\int \cos A u^2 du = \varphi$ , on trouve :

$$I = C^2 (\theta^2 + \varphi^2) \quad (14)$$

Les intégrales  $\theta$  et  $\varphi$  se ramènent aux intégrales de Fresnel

$$\eta = \int_0^v \sin \frac{\pi}{2} v^2 dv \text{ et } \xi = \int_0^v \cos \frac{\pi}{2} v^2 dv \text{ en posant } A u^2 = \frac{\pi}{2} v^2. \text{ Il vient, en tenant compte}$$

de ce que  $\sqrt{\frac{2A}{\pi}} \left( h + \frac{y_1}{2} \right)$  est grand et de la nature particulière des fonctions  $\eta$  et  $\xi^2$  :

$$\begin{aligned} I &= \frac{\pi C^2}{2A} \left[ \left( \int_{-\infty}^{\sqrt{\frac{A}{2\pi}} y_1} \sin \frac{\pi}{2} v^2 dv \right)^2 + \left( \int_{-\infty}^{\sqrt{\frac{A}{2\pi}} y_1} \cos \frac{\pi}{2} v^2 dv \right)^2 \right] \\ &= \frac{I_0}{2} [(\eta_{v_1} - \eta_{-\infty})^2 + (\xi_{v_1} - \xi_{-\infty})^2] \quad (y_1 < 0) \end{aligned} \quad (15)$$

\* La solution de cette intégrale nous a été aimablement communiquée par Monsieur J. TRIS, Aspirant au F.N.R.S.



Si l'on supprime le couteau, les limites d'intégration deviennent  $-\infty$  et  $+\infty$  et la somme des termes entre crochets dans (15) devient égale à 2. L'intensité  $I$  en  $y$ , étant alors égale à  $I_0$ , le coefficient  $\frac{\pi C^2}{2A}$  doit donc être de la forme  $\frac{I_0}{2}$ .

L'expression (15) est complètement analogue à celle obtenue dans le cas général de la diffraction par un écran à bord droit. On peut montrer aisément en utilisant une spirale de CORNU<sup>2</sup> que:

1. l'ombre géométrique correspond à une intensité  $\frac{I_0}{4}$ ;
2. le second membre de (15) est maximum ou minimum respectivement pour des valeurs de  $v_k$  et  $v'_k$  données par les formules:

$$v_k = \sqrt{4k - 2.5} = \sqrt{\frac{A}{2\pi}} y_k \text{ et } v'_k = \sqrt{4k - 0.5} = \sqrt{\frac{A}{2\pi}} y'_k \quad k = 1, 2, 3 \dots \quad (16)$$

Les ordonnées  $y_k$  pour lesquelles on observera un maximum d'intensité sont:

$$y_1 = \frac{1.22}{\sqrt{\frac{A}{2\pi}}}, y_2 = \frac{2.34}{\sqrt{\frac{A}{2\pi}}}, y_3 = \frac{3.08}{\sqrt{\frac{A}{2\pi}}}, \dots y_k = \frac{\sqrt{4k - 2.5}}{\sqrt{\frac{A}{2\pi}}} \quad (17)$$

Les ordonnées pour lesquelles on observera un minimum d'intensité sont:

$$y'_1 = \frac{1.87}{\sqrt{\frac{A}{\pi 2}}}, y'_2 = \frac{2.74}{\sqrt{\frac{A}{2\pi}}}, y'_3 = \frac{3.40}{\sqrt{\frac{A}{2\pi}}}, \dots y'_k = \frac{\sqrt{4k - 0.5}}{\sqrt{\frac{A}{2\pi}}} \quad (18)$$

On voit immédiatement que l'écartement des franges dépend de  $A$ , c.à.d. du gradient, et qu'elles sont d'autant plus serrées que le gradient est plus élevé.

Il nous reste maintenant à calculer numériquement les écartements et par conséquent à déterminer la valeur de  $\sqrt{\frac{A}{2\pi}}$ .

On sait que le gradient d'indice définissant une frontière de migration est en réalité de la forme<sup>3</sup>:

$$n'(\bar{y}) \equiv \frac{dn}{d\bar{y}} = \frac{\Delta n}{2\sqrt{\pi Dt}} e^{-\frac{\bar{y}^2}{4Dt}} \quad (19)$$

Dans cette expression, le sommet de la courbe est en  $\bar{y} = 0$ ;  $\Delta n$  est la différence d'indice entre les deux milieux extrêmes;  $D$  est la constante de diffusion de la protéine responsable de la limite;  $t$  est le temps en secondes.

La courbe intégrale de (19) est une courbe en S. Rappelons que nous avons choisi comme répartition des indices dans la cellule une parabole représentée par l'équation (2). Nous pouvons maintenant considérer cette parabole comme étant la parabole osculatrice à la courbe intégrale de (19) et écrire:

$$\frac{d^2n}{dy^2} \equiv \frac{-\bar{y} \frac{du}{d\bar{y}}}{2Dt} = -2a \quad \text{d'où} \quad a = \frac{\bar{y}n'(\bar{y})}{4Dt} \quad (20)$$

La constante A est définie par les relations (9) et (10):

$$A = \frac{4\pi ea}{\lambda} \quad \text{et} \quad \frac{A}{2\pi} = \frac{2ea}{\lambda} \quad (21)$$

Si  $\beta$  est l'angle que fait avec l'axe y la tangente à la courbe de gradient (19), on voit immédiatement que:

$$2a = \operatorname{tg} \beta \quad (22)$$

$\operatorname{tg} \beta$  se calcule facilement par une mesure directe sur les clichés en tenant compte des constantes de l'appareil.

#### B. CALCULS NUMÉRIQUES ET VÉRIFICATION EXPÉRIMENTALE

1. Les relations (17), (18), (21) et (22) permettent une vérification immédiate de la théorie.

Les relations (17) donnent les rapports caractéristiques suivants:

$$\frac{y_2 - y_1}{y_3 - y_1} = 0.60, \quad \frac{y_2 - y_1}{y_4 - y_1} = 0.45, \quad \frac{y_2 - y_1}{y_5 - y_1} = 0.38 \quad (23)$$

En tenant compte de (17), (18), (21) et (22), on voit que les écartements des franges pris à deux niveaux d'une courbe de gradient et caractérisés par deux valeurs différentes de  $\operatorname{tg} \beta$ , sont entre eux comme la racine carrée de l'inverse du rapport des tangentes:

$$\frac{(y_2 - y_1)_1}{(y_2 - y_1)_2} = \frac{(y_3 - y_1)_1}{(y_3 - y_1)_2} = \dots = \sqrt{\frac{\operatorname{tg} \beta_2}{\operatorname{tg} \beta_1}} \quad (24)$$

Les tableaux ci-dessous rassemblent quelques mesures faites sur des gradients réels:

1.

$y_2 - y_1, y_3 - y_1, \dots \cdot 10^{-1} \text{ mm}$				$\frac{y_2 - y_1}{y_3 - y_1}, \frac{y_2 - y_1}{y_4 - y_1}, \dots$		
6	10	13.5	—	0.60	0.45	—
3.5	6	7.5	9	0.58	0.47	0.39
4	6.5	9	11	0.62	0.45	0.36
7.5	13	17	—	0.58	0.44	—

2.

$\frac{(y_2 - y_1)_1}{(y_2 - y_1)_2}$	$\frac{7.5}{3.5} = 2.4$	$\frac{7.5}{5.0} = 1.5$	$\frac{9.0}{6.0} = 1.5$	$\frac{12}{6.0} = 2.0$	$\frac{8.0}{6.0} = 1.3$
$\sqrt{\frac{\operatorname{tg} \beta_2}{\operatorname{tg} \beta_1}}$	$\sqrt{\frac{38.2}{7.1}} = 2.3$	$\sqrt{\frac{22.9}{7.2}} = 1.8$	$\sqrt{\frac{8.1}{4.3}} = 1.4$	$\sqrt{\frac{19}{5.4}} = 1.9$	$\sqrt{\frac{22}{14}} = 1.25$

On voit, d'après ces deux tableaux, que la théorie développée plus haut est en très bon accord avec les résultats expérimentaux. La mesure directe des distances  $y_2 - y_1$ ,  $y_3 - y_1$  etc. permet de calculer immédiatement la position du bord géométrique; il est donc possible de tracer le contour exact du gradient à partir de la position de deux franges facilement repérables au moyen d'un simple compas de proportion.

Reprenons par exemple le microphotogramme des franges publié par LONGSWORTH (Fig. 3). L'auteur situe l'ombre géométrique en  $h_0$ , sensiblement à une intensité caractérisée par  $\frac{d_0}{4}$  où  $d_0$  est le noircissement. La mesure de l'écartement des franges montre



Fig. 3. Microphotogramme des franges de diffraction (d'après LONGSWORTH<sup>1</sup>)

qu'en réalité l'ombre géométrique correspond presque exactement à la limite ombre-lumière enregistrée, soit H sur la figure. Les écarts mesurés à partir de  $h_0$  sont en effet pour  $y_1, y_2, y_3$ : 0.7, 1.7, 2.3. Les relations (23) sont satisfaites, mais les équations (17) ne se vérifient qu'à condition de placer le bord géométrique en H; les écarts  $y_1, y_2, y_3$  deviennent alors 0.7 + 0.3, 1.7 + 0.3, 2.3 + 0.3. L'ombre géométrique correspond à un noircissement déterminé, mais qui dépend des caractéristiques du matériel photographique, du choix du temps de pose et des conditions de développement.

2. Les équations (17) à (22) peuvent aussi servir à calculer la valeur absolue des écartements.

a. Ecartements calculés d'après les clichés expérimentaux, au moyen des équations (22), (21) et (17):

Constantes: distance couteau-cellule corrigée pour le trajet dans la cuve thermostatique:  $R = 100$  cm;  $e = 0.6$  cm;  $\lambda = 5 \cdot 10^{-5}$  cm. Rapport déplacement couteau/plaque: 1/7 ou 1/14;  $\frac{I}{C}$  grandissement:  $G = 0.875$ .

$\beta'$	$\text{tg } \beta'$	$a = \frac{\text{tg } \beta'}{2 \text{ Rec}}$	$\frac{A}{2\pi} = 2,4 \cdot 10^4 a$	Ecartements calculés		Ecartements observés $\times \frac{I}{G}$	
				$y_1$	$y_2$	$y_1$	$y_2$
61°	1.80	$\frac{0.9}{420}$	51	0.170	0.33	0.17	0.34
88°30'	38.2	$\frac{19.1}{420}$	1090	0.037	0.07	0.035	0.07
87°	19	$\frac{9.5}{840}$	270	0.075	0.14	0.07	0.135

L'accord est aussi bon que possible.

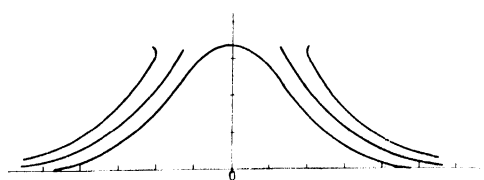
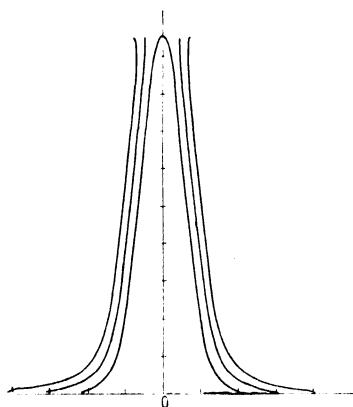
b. Courbes de gradient et franges calculées d'après les équations (19), (20) et (17).

Constantes:  $D = 5 \cdot 10^{-7}$ ,  $\Delta n = 0.0043$ ,  $R = 100$  cm,  $\frac{I}{C} = 1/7$ ,  $e = 0.5$  cm,  $\lambda = 5 \cdot 10^{-5}$  cm, grandissement 1.

Les Figures 4 et 5 montrent les courbes de gradients et la disposition des franges pour des temps  $t = 36000$  et  $288000$  sec.

L'allure des phénomènes de diffraction est semblable à celle observée sur les clichés réels (Figs 6 et 7).

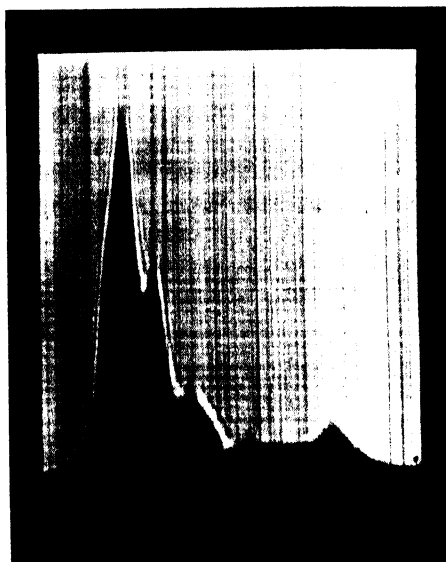
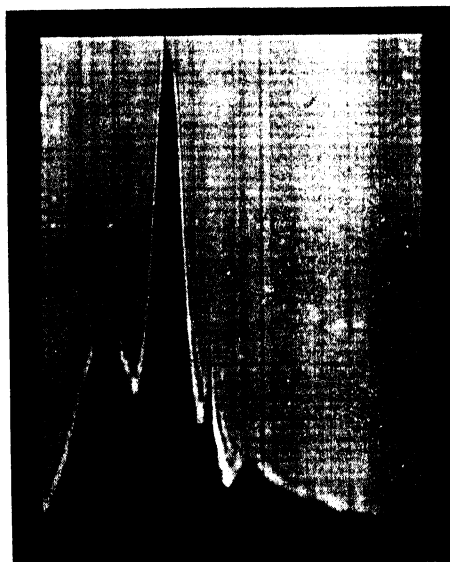
On voit que les franges s'écartent légèrement au sommet et que leur disparition



Figs 4 et 5. Courbes de gradients et franges théoriques

permet de situer exactement le point de gradient maximum. Pour des gradients très petits, la présence de franges est un indice certain de l'existence de ces gradients.

Ajoutons, pour terminer, quelques mots sur l'importance des fentes utilisées dans l'appareil. Les franges et la limite de l'ombre sont d'autant mieux définies que les fentes sont étroites. Une fente large à la source lumineuse produit une superposition des contours et des franges, légèrement décalés les uns par rapport aux autres. Cet effet est surtout important, là où la variation du gradient est faible, c'est-à-dire au



Figs 6 et 7. Clichés expérimentaux d'électrophorèses de protéines musculaires. Appareillage du type LONGSWORTH décrit par DUBUISSON ET JACOB<sup>4</sup>

sommet et à la base des contours. L'ombre géométrique ne correspond plus aux points  $\frac{I_0}{4}$ , mais pourra encore être précisée d'après la position des franges si celles-ci sont visibles. On règlera la dimension des fentes d'après les clichés.

### C. CONCLUSIONS

Les phénomènes de diffraction observés sur les clichés d'électrophorèse à l'aide de l'appareillage décrit par LONGSWORTH rentrent dans le cadre général de la diffraction de la lumière par un écran à bord droit. Ils en forment un cas particulier intéressant. On peut, en utilisant les équations que nous avons établies, calculer la position des franges en fonction du gradient observé.

Le contour exact des courbes de gradients est défini par le lieu des points d'intensité  $\frac{I_0}{4}$ . On peut, par une mesure directe de l'espacement des franges, vérifier si la limite ombre-lumière observée sur les clichés correspond bien au contour théorique. Eventuellement, le contour peut être tracé par points, à partir d'un repérage précis des franges.

Nous tenons à remercier bien vivement Monsieur F. TEMMERMAN, Ing. A. I. Br, pour l'enseignement et l'aide considérable qu'il nous a prodigués lors de l'élaboration de ce travail.

### RÉSUMÉ

La position des franges de diffraction observées sur les clichés d'électrophorèse du type LONGSWORTH peut être calculée, en excellent accord avec l'expérience, en fonction du gradient observé et des constantes de l'appareil. Le phénomène est un cas particulier de la diffraction par un écran à bord droit, mais où l'écartement des franges est fonction du gradient. Le repérage précis des franges peut permettre de tracer par points le contour exact de la courbe de gradients.

### SUMMARY

The positions of the diffraction bands observed on LONGSWORTH electrophoresis photographs can be calculated in excellent agreement with the experimental results from the gradient observed and the constants of the apparatus. This phenomenon is a special case of diffraction by a screen with straight edges, where, however the interval between the bands is a function of the gradient. By marking the precise position of the bands the curve of the gradients can be drawn point by point.

### ZUSAMMENFASSUNG

Die Lage der Diffraktionsbanden, welche man auf den photographischen Aufnahmen einer im LONGSWORTH'schen Apparate ausgeführten Elektrophorese beobachtet, können in ausgezeichneter Übereinstimmung mit den Versuchsergebnissen aus dem beobachteten Gradienten und den Konstanten der Apparatur berechnet werden. Diese Erscheinung ist ein besonderer Fall der Diffraktion durch einen Schirm mit geradem Rand, wo aber die Entfernung der einzelnen Banden vom Gradienten abhängt. Zeichnet man die Banden genau ein, so findet man punktweise den genauen Verlauf der Kurve der Gradienten.

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## X-RAY DIFFRACTION STUDIES OF HUMAN CHORDAE TENDINEÆ\*

by

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When an X-ray beam is passed through material having a periodic structure, it is diffracted in a characteristic fashion according to the dimensions of the crystalline lattice of the material, the degree of crystallinity, and the amount of orientation of the component molecules. Diffraction studies have yielded knowledge of the intimate structure of many organic and inorganic compounds and varieties of biological material.

The diffraction pattern of collagen is well established. The three characteristic spacings obtained in large-angle diffraction studies are of approximately 2.8, 4 and 10 Angstrom units, and are considered<sup>1</sup> to represent respectively the average amino acid residue length, the average backbone spacing, and the average side-chain distance of the collagen molecules. The presence of these three spacings is sufficient to identify the material as collagen, and no matter what its source—tunica albuginea, tendon, scar tissue, chorda tendinea, etc.—there is little variation in the pattern. X-ray diffraction studies can also furnish information as to the degree of orientation of the molecules; complete circles or discrete arcs represent lesser or greater orientation respectively. The degree of crystallinity is indicated by the width of the rings themselves, a thicker ring appearing with a specimen of low crystallinity.

Studies have been undertaken in this laboratory to determine whether alteration of the X-ray diffraction pattern of human chordae tendineae accompanies aging. This tissue was selected because it is easy to obtain and to prepare, consists almost entirely of connective tissue, and is under continual stress during life.

*Method:* At routine autopsies, groups of chordae tendineae were removed from the mitral valve, together with contiguous portions of valve and papillary muscle. In the presence of valvular disease or deformation of the chordae, the specimen was discarded. Following 20% formalin fixation, the tissue was washed in water, and a single chorda approximately 100 to 150 microns in thickness removed, dried on glass without tension, and mounted over the collimating hole in the microcamera so that the beam would pass through the center of its axis. In the microcamera used in these investigations\*\*\*, the collimated beam was about 60 by 80 microns in width, the film to specimen distance 13.4 mm, the film diameter about 43 mm. Copper K-alpha radiation (1.54 Angstrom

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\*\* Sarah Welt Fellow in Medicine, 1947; Research Fellow, American Cancer Society, 1948.

\*\*\* The camera was built in this laboratory, and is identical in all essential features to the micro-camera designed by FANNKUCHEN. A similar camera has been recently described by F. G. CHESLEY (X-ray Diffraction Camera for Microtechniques, *Rev. Sci. Instr.*, 18 (June, 1947) 422-424.

units) was used, with Eastman Kodak No-Screen film and an exposure time of about 10 hours.

Preliminary studies revealed that the degree of orientation of the diffraction pattern was unaltered whether the specimen was fixed or unfixed, the exposure time long or short. When the beam passed through the periphery of the chorda, the pattern was less distinct but otherwise identical with that formed when the beam passed through the center of the axis. Repeated examinations along the same chorda, exposures of several chordae from mitral and tricuspid valves of the same subject and of chordae of various thicknesses from the same subject, all failed to show significant variations. The classical collagen X-ray diffraction pattern is illustrated schematically in Fig. 1.

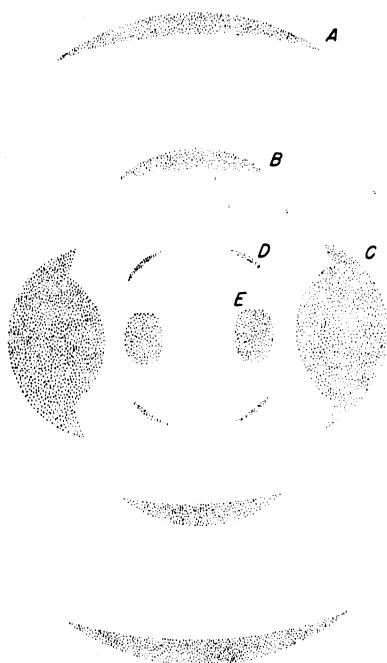


Fig. 1. Schematic X-ray diffraction pattern of oriented collagen.

Patterns were arbitrarily placed in three categories, "poor", "medium", and "good", depending on the degree of orientation. With "good" orientation, A, B, C, D and E (Fig. 1), are distinct, as in Fig. 2. In "poor" orientation (Fig. 4), A appears as a complete circle, B and C form the uniform middle ring, and D and E comprise the inner circle, with D usually merging with the background. In "medium" orientation (Fig. 3), A appears as a larger arc, C appears as meridional accentuation, E appears as arcs, and D is not discernible\*.

*Results:* Data are summarized in Table I, which is a listing, by chronological age of the subjects, of the degree of orientation of the chordae tendineae examined. It is to be noted that with very few exceptions the best orientation was found in the older subjects. Higher orientation indicates a higher degree of alignment of collagen molecules in the axis of the chordae. Whether this is a result of constant tension, reorientation or reorganization of connective tissue as part of the aging process, or some process not heretofore recognized, remains to be determined. It appears that during adolescence the maximum degree of orientation is achieved, as far as can be inferred from the arrangement of data

in our three groups. A quantitative measure of orientation by densitometric measurements is under consideration. Preliminary results, however, do not appear promising. It may be pertinent that CLARK AND ZIEGLER<sup>2</sup>, studying the X-ray diffraction patterns of catgut ligatures, correlated increased tensile strength with higher orientation. Studies are now being undertaken to correlate the histological appearance of the chordae with the diffraction pattern. In addition, it is planned to investigate other collagenous structures of the body from the standpoint of the X-ray diffraction pattern at various ages during health and disease.

\* The outer arc A in Fig. 3 and the corresponding ring in Fig. 4 is not visible in the half tone reproductions; they are quite distinct in the original films.



Fig. 2. X-ray diffraction pattern of chordae tendineae of a 70 year old adult, showing "good" orientation





Fig. 3. X-ray diffraction pattern of chordae tendineae of a 45 year old adult, showing "medium" orientation

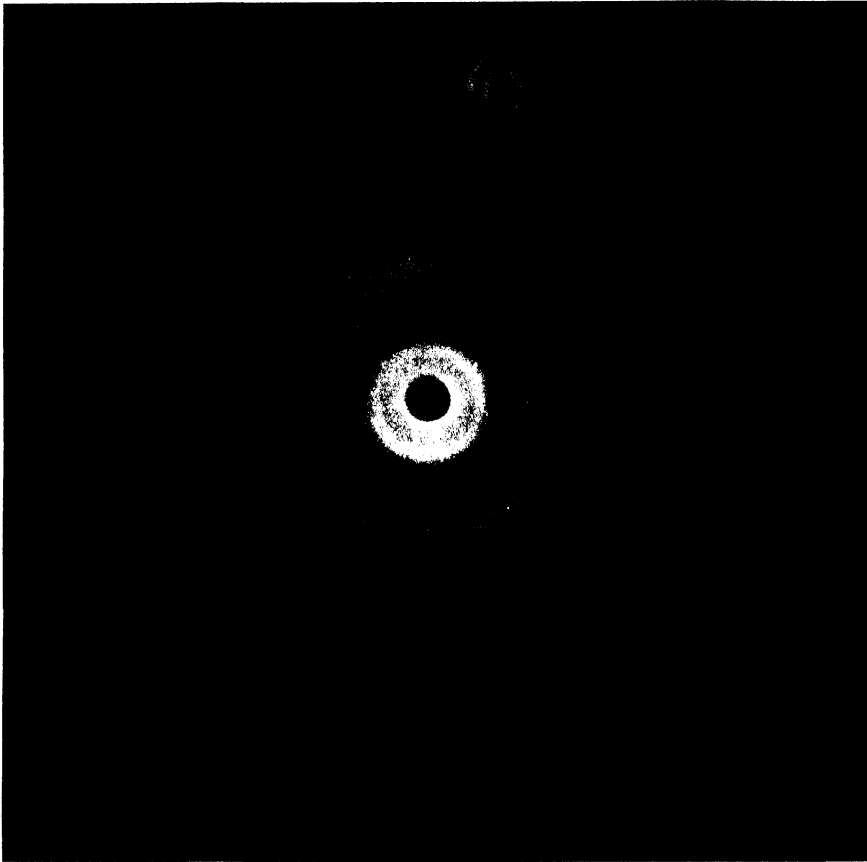


Fig. 4. X-ray diffraction pattern of chordae tendineæ of a year old infant, showing "poor" orientation

Table 1

ORIENTATION OF THE X-RAY DIFFRACTION PATTERN OF THE CHORDAE TENDINEÆ OF HUMANS AT VARIOUS AGES, ARRANGED IN CHRONOLOGICAL ORDER

Age	Orientation	Age	Orientation	Age	Orientation	Age	Orientation
Premature	Poor	5 months	Poor	42 years	Good	70 years	Good
Premature	"	5 months	"	44 years	Medium	70 years	"
Premature	"	2 years	Medium	45 years	Good	72 years	"
Newborn	"	4 years	Poor	61 years	"	72 years	"
Newborn	"	4 years	Medium	62 years	"	72 years	"
Newborn	"	4 years	Poor	65 years	"	73 years	"
3 weeks	"	35 years	Good	65 years	"	74 years	"
4 weeks	"	35 years	"	69 years	"	74 years	"

## SUMMARY

X-Ray diffraction patterns of chordae tendineæ in 32 autopsy cases in subjects of varying age show a poor orientation of collagen molecules in the younger age groups (below four years) and an increasing degree of orientation in older age groups (all subjects over 45 years show a high degree of orientation).

## RÉSUMÉ

Les diagrammes de diffraction des rayons X des chordae tendineæ prélevées dans 32 cas d'autopsie chez des sujets d'âges différents, montrent que l'orientation des molécules de collagène est peu marquée chez les individus les plus jeunes (en-dessous de 4 ans) et que cette orientation s'accroît avec l'âge (tous les individus âgés de plus de 45 ans présentant un degré élevé d'orientation).

## ZUSAMMENFASSUNG

Röntgenstrahlinterferenzbilder von chordae tendineæ in 32 Autopsien zeigen geringe Gleichrichtung der Kollagenmoleküle bei Individuen unter 4 Jahren und ein Zunehmen dieser Gleichrichtung mit dem Alter (in allen Fällen über 45 Jahre wurde ein hoher Grad der Gleichrichtung gefunden).

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## SOME EXPERIMENTS ON THE ORIENTATION AND HARDENING OF KERATIN IN THE HAIR FOLLICLE

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### INTRODUCTION

The synthesis of a fibrous and keratinized protein is known to occur in several steps but these need fuller characterization. The experiments described below were carried out to enable the development of the oriented fibrous structure and the hardening process to be followed more precisely.

The experimental material has been principally the hair follicle from the human head. This choice was suggested by the ease of obtaining suitable specimens by plucking, by the fact that the pertinent histological structures are easily visible, and because the various stages in the development are conveniently set out in an orderly sequence along the lumen of the follicle. In spite of the diversity of the epidermal appendages containing keratin as their characteristic protein, it is probable that the course of synthesis is similar in each and that general conclusions may be drawn from the study of a particular case such as hair.

The histochemical studies of GIROUD AND BULLIARD<sup>1</sup> showed that a pre-keratin, characterized by the presence of free thiol (SH) groups, precedes the formation of keratin proper, and that hardening involves the oxidation of the thiol groups to produce disulphide bridges between the peptide chains.

This is in harmony with chemical observations on wool and hair<sup>2, 3</sup>, which relate the physical toughness and chemical inertness of the protein to the high proportion of cross links between the chains made possible by the cystine residues. MARSTON<sup>4</sup> later showed that the oxidative system needs copper to be effective. In the absence of traces of copper the keratinization is delayed and incomplete. Using strong solutions of urea, RUDALL<sup>5</sup> extracted from the germinal layers of skin a protein which was probably the pre-keratin or a derived protein. He showed that it was a typical  $\alpha$ -type protein, capable of yielding both  $\alpha$ - and  $\beta$ -type X-ray patterns and displaying long range elasticity when in the form of fibres. In most of its properties it behaved as might be expected from a keratin deficient in cross-linking. Some of the experiments described here were suggested by analogous experiments carried out by RUDALL<sup>5, 6</sup> on the epidermal protein.

The histology of the hair follicle is well known and reference may be made to the standard texts of MAXIMOV<sup>7</sup> and SCHMIDT<sup>8</sup>. The cells destined to form the fibre and its attendant sheaths originate in the proximity of the papilla. In their progress along the follicle they differentiate to form the structures of the hair and sheaths. The optical properties of the final hair, as was shown by SCHMIDT, are equivalent to those of an uniaxial crystal. The birefringence of dry hair is of the order 0.011–0.013<sup>8, 9</sup>. The pre-

cortex, cuticles and sheaths are also birefringent in differing degrees, and are therefore easily distinguished without staining by using a polarizing microscope. In this paper, however, discussion will be limited to the development of the cortex, since here the characteristic keratin of the fibre is formed<sup>11, 12</sup>. The presumptive cortical cells in the vicinity of the papilla are roughly spherical in shape. At the constriction of the bulb they elongate and acquire the long spindle shape which persists in the final fibre. At the time of elongation the cells become birefringent and this feature suggested the use of polarized light as an adjunct to the histochemical procedures. The principal features indicated by the birefringence measurements were also confirmed by X-ray photographs made at different levels of a single follicle.

#### EXPERIMENTAL

##### *Experimental material and birefringence measurements*

Hairs plucked from the human head usually bear with them the inner root sheath and all the included structures of the hair root. The plucking causes no perceptible distortion, as may be seen by comparing the plucked root with those sectioned *in situ*. The specimens used in this work were non-medullated and lightly pigmented. The final hairs were about  $50\ \mu$  in diameter. Fresh, unfixed and unstained roots were needed for most experiments. Birefringence measurements were made by means of a Sénarmont

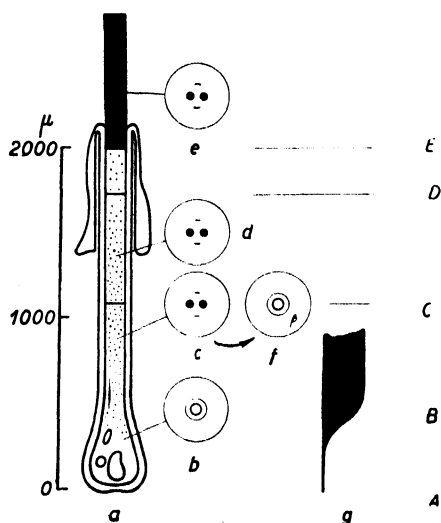


Fig. 1. Some features of the freshly plucked hair root as referred to in the text. The inner root sheath envelopes the developing fibre. The several zones of the presumptive cortex are: AB the isotropic zone of the bulb, B the level of fibrillation, BC the unconsolidated zone, CDE the zone of hardening (keratinization). The region BE shown dotted is the pre-keratinous region giving a positive reaction with the nitroprusside reagent. At b, c, d and e are the X-ray patterns obtained at the levels shown. At f is the disoriented  $\beta$ -pattern obtained from BC after heating. At g the development of birefringence is shown in relation to the various levels.

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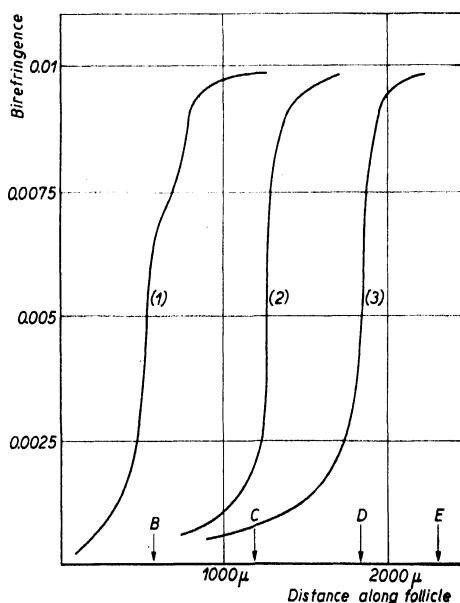


Fig. 2. The relation between the total birefringence of the pre-cortex of the hair follicle and the distance along the follicle from the base. (1) Development of birefringence in the freshly plucked root. (2) After heating in mounting oil for 30 seconds at  $90^\circ\text{C}$ . The orientation in the lower half of the pre-keratin has been destroyed. See also Fig. 1. (3) After treatment with 0.05 N sodium hydroxide for 2 min. The letters B, C, D, and E indicate the same levels as in Fig. 1.

compensator mounted on a microscope, and diameters by means of a micrometer eyepiece. The bulb region AB (Fig. 1) is almost isotropic<sup>8</sup>. As may be seen from Fig. 2, Curve 1, the development of birefringence begins suddenly at the level of the constriction B. The full degree of orientation is established rapidly at the beginning of the pre-keratinous zone BE.

An attempt to assess the amount of solid material, presumed to be wholly protein, at the several levels of the root was made by drying over phosphorous pentoxide and comparing the diameters at successive levels before and after drying. Some assumptions were necessary: that cellular movement in bulk did not occur on drying, and that the dry root was fully and uniformly collapsed and had a uniform density along its length. The results are probably reliable enough for the present purpose and receive support

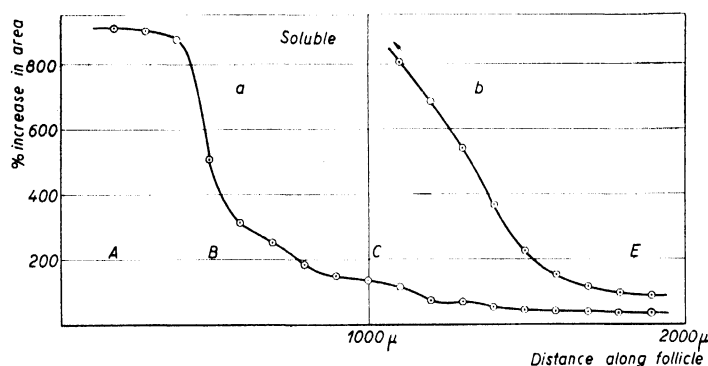


Fig. 3. The swelling of the hair root: (a) in water (b) in saturated urea. In the latter case the lower portion BC of the pre-cortex dissolves, and the progressive hardening of the protein above C is shown by the decrease in swelling. Other lettering as in previous figures.

from other considerations. The dry solid content of the root was found by this means to be the same at all levels from about 100  $\mu$  above the top of the papilla. The primary synthesis thus seems to be largely complete before the conversion into the fibrous state begins. The degree of hydration of the cells was also obtained by difference between the wet and dry areas. A very rapid dehydration occurs at the constriction of the follicle.

#### *Histochemical observations*

The fully hardened fibre is stable in boiling water but contracts with a marked fall in birefringence on raising the temperature to 130° C<sup>13</sup>. The fibre then gives a typical disoriented  $\beta$ -X-ray pattern. Reagents not capable of attacking disulphide bonds have little effect on hair. The course of consolidation may therefore be followed by determining the increase in resistance to temperature, to enzymes, and to such reagents as dilute acids, alkalis, and solutions of urea, as the follicle is ascended. The nitroprusside reaction used by GIROUD AND BULLIARD<sup>1</sup> shows the presence of unoxidized thiol groups in the whole region BE, referred to as the pre-keratinous zone. The reaction is not of great use in the quantitative sense. Birefringence measurements were most useful for detecting and following the course of reactions which often produce no other visible change. Staining techniques were not found very useful. Many dyes enter the pre-keratinous zone first and later stain more uniformly. This is probably a question of diffusion only.

a. *Enzymes.* 0.1 % trypsin at pH 8 and 40° C rapidly digested the cytoplasmic protein of the bulb and in about two hours removed the pre-cortex to a level some 1100  $\mu$  from the base, i.e., the zone marked BC in Fig. 1 and Fig. 4b. The inner root sheath may be seen to be more resistant and appears to harden first at the level B.

b. *Dilute acids and bases.* Hydrochloric acid 2N penetrates the whole of the pre-keratinous zone in about a minute, as may be seen by staining with an indicator before treatment. Subsequent to penetration a fall in birefringence occurs in the same region BC as is also preferentially digested by trypsin. BC will be referred to as the unconsolidated zone. In the course of a few minutes the birefringence falls nearly to zero, as is shown in Fig. 2, but the protein does not actually dissolve in this time. Caustic soda 0.05 N destroys the birefringence to a higher level D and ultimately dissolves the protein. The nitroprusside reaction is still positive for a short length DE above the level removed by alkali, showing that something short of the complete degree of hardening makes the protein resistant to alkali.

c. *Solutions of urea* are very effective in disorganizing and dissolving the unconsolidated zone BC (Fig. 4c). This observation parallels that made by RUDALL<sup>5</sup> on the analogous layers of the skin. Saturated urea often attacks at the higher level C as can be seen in Fig. 4c, but this may be a result of plucking. Experiments on roots *in situ* in cow's skin failed to show the effect. Electron microscopic examination of the dispersed material after dialysis showed only a confused agglomeration of particulate matter and no fibrous structure.

d. *Heat.* The birefringence of the unconsolidated zone was also destroyed by heating at 90–95° C for 30 sec in water or in mounting oil (Fig. 4a). No contraction in length of this region was noticed, which is in contrast to the contraction that occurs on heating the fully keratinized hair to 130° C, and may mean that the fibrous structure at this level lacks a strong system of longitudinal covalent links. The X-ray photograph obtained from the zone BC after heating was the fully disoriented  $\beta$ -type (Fig. 5c).

The various zones demonstrated in the above experiments: the isotropic bulb, the fibrillation zone at B, the unconsolidated fibrous zone BC, and the zone of progressive hardening CE, are also distinguished by different degrees of swelling in water as shown in Fig. 3a.

By dissecting out the various levels and repeating the experiments on the isolated pieces, it was shown that none of these effects was due to any peculiarity of the sheaths or cuticles.

e. *Effect of reduction and of cross-linking.* The length of the unconsolidated region can be increased by subjecting the root to reduction. After 30 min treatment with zinc and hydrochloric acid (saturated with salt to suppress swelling) it was found that heating now destroyed the birefringence to a level about 200  $\mu$  above C. Longer treatment stabilized the lower zone BC and may indicate the introduction of cross links derived from the metallic ions. Thioglycollate solutions were also effective but were very destructive of the sheaths, making observation difficult. RUDALL<sup>5</sup> showed that formalin and other cross-linking reagents were capable of stabilizing the protein isolated from the skin. Similarly in the case of the hair root 10 min treatment in 20% formaldehyde solution destroys the thiol reaction and at the same time hardens the protein and renders the oriented structures stable to heating to 100° C for at least 5 min. The total value of the birefringence and its course of development were not altered by the introduction of such cross links. Treatment with ethylene dibromide in the presence of sodium bicar-

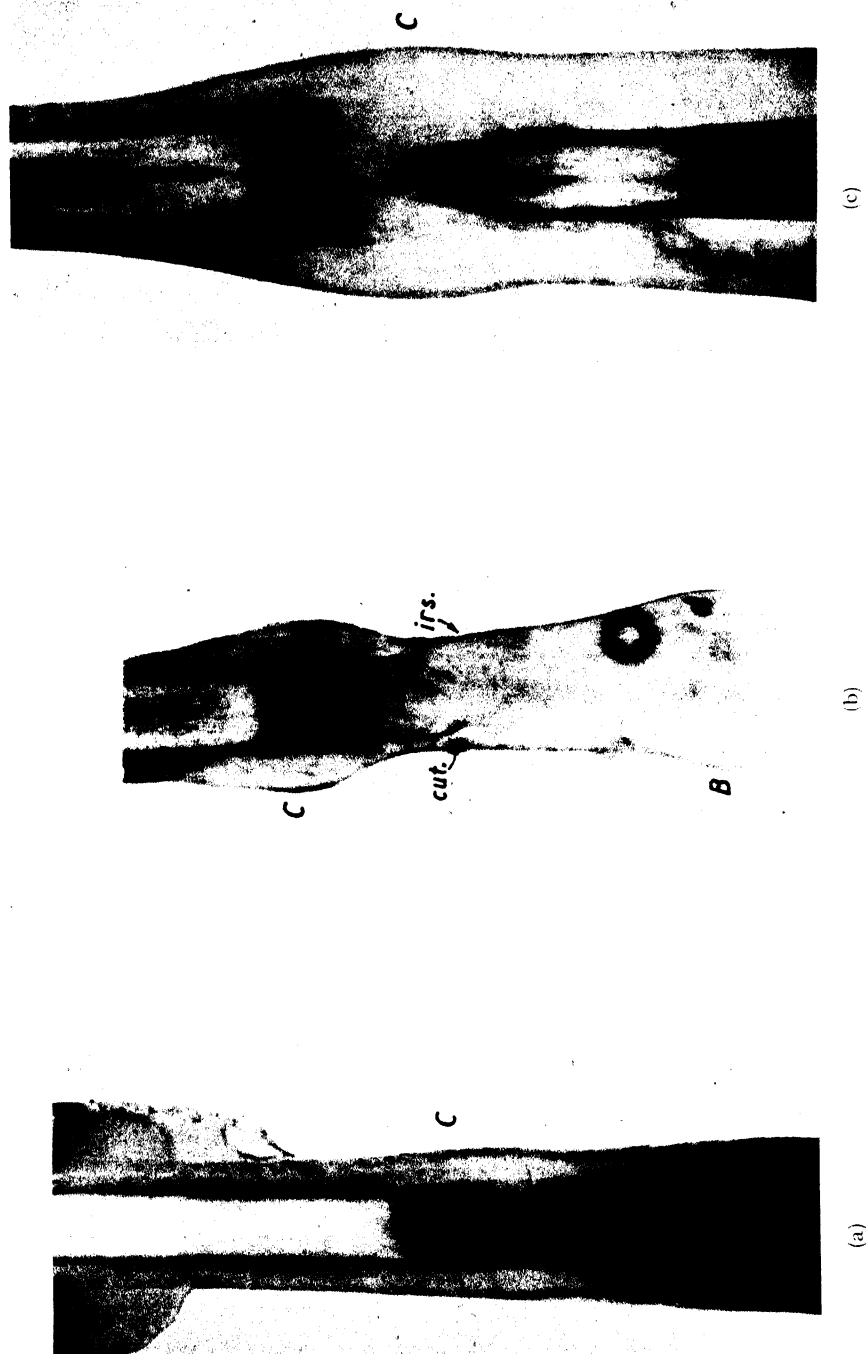


Fig. 4. Behaviour of hair follicles in experiments designed to show the instability of the unconsolidated zone BC. Polarized light and a Sénarmont compensator have been used to define the features more clearly. (a) Central portion of a hair root after heating for 1 minute at 90°C. The birefringence has been destroyed to a level C. (b) The result of digesting a follicle in trypsin for 2 hours. The unconsolidated zone BC has been removed. The inner root sheath (i.r.s.) and the fibre cuticle (cut.) are relatively more resistant. (c) The action of saturated urea. Photograph taken about 2 minutes after action had commenced. The zone BC is in the act of being dispersed, but the partly keratinized protein above C only swells.



bonate, which is known to unite reduced disulphide groups in wool<sup>14</sup>, also stabilizes the lower zones but is not as effective as formaldehyde. Direct oxidation by means of 2% hydrogen peroxide had a small effect, but destruction of the sheaths again made observation difficult.

### *X-ray observations*

Exposures were made to determine the orientation of the crystalline portion of the fibre and also to characterize the protein. Micro-X-ray photographs were obtained from a single root at those levels, shown in Fig. 1, which the previous birefringence observations had shown to be of special interest. A collimator 0.01 cm in width, about the same as the

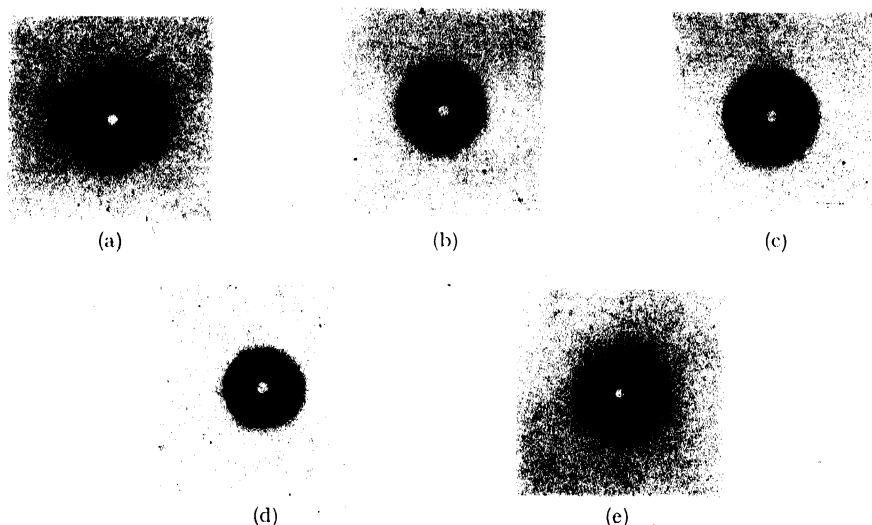


Fig. 5. X-ray photographs obtained from the several levels of a single hair root. Specimen to film distance 1 cm. (a) From the bulb. (b) The unconsolidated zone BC. (c) The unconsolidated zone BC after disorientation by heat. Disoriented  $\beta$ . (d) The partly consolidated zone. (e) The fully hardened hair above E. b, d and e are typical  $\alpha$ -patterns.

diameter of the root itself, and 4 cm long was used. With filtered Cu K $\alpha$  radiation from a continuously evacuated filament tube, exposure times of 25–30 h were needed when the specimen to film distance was 1 cm. The specimen was hardened in formalin (10% for 10 min) to minimize changes during the long exposures and was photographed dry. In conformity with the birefringence findings, which show that the pre-cortex becomes fully oriented at the fibrillation level B, well oriented patterns of the  $\alpha$ -type, characteristic of keratin<sup>11</sup>, were obtained at all levels save the lowest, that of the bulb. The photograph of the bulb showed two diffuse rings typical of disoriented native proteins. After heating the follicle to 90° for one minute, the pattern given by the zone BC was a typical disoriented  $\beta$ .

Photographs of the several levels are reproduced in Fig. 5(a–e).

### DISCUSSION

The experiments described enable several regions of different stability to be distinguished in the presumptive cortex of the developing hair. These are 1. *the isotropic bulb*

region, Fig. 1 AB. The birefringence is very low and the X-ray photograph shows an absence of oriented crystalline material. It is difficult to determine the actual site of synthesis of the protein which is destined to become keratin, but the observations on the cross-sectional areas of dried roots suggest that considerable amounts of solid material are present in the upper part of the bulb where the birefringence is still low.

2. *The fibrillation level B.* The appearance of fibrils, the sudden rise in birefringence, the contraction in the width of the follicle, and the dehydration of the cells all occur at the same level and are probably causally connected.

3. *The unstabilized fibrous region BC.* The X-ray pattern is of the  $\alpha$ -type, *i.e.*, apparently the same as that of the final hair, and the birefringence is also nearly the same. But the fibre is as yet poorly consolidated and is readily disoriented with the production of a disoriented  $\beta$ -type structure.

4. *The consolidation zone (keratinization) CDE.* The hardening of the protein appears to begin quite definitely at a point about 700  $\mu$  above the level of fibrillation. The total birefringence and the X-ray pattern are not affected by the changes which ensue. The consolidation is progressive and leads to the fully hardened region above E.

An account in molecular terms of the sequence of events which lead to the oriented and hardened fibre would be of great interest but we are handicapped at the very start by lack of knowledge of the initial state of the protein in the cells of the bulb. The molecules of the primary protein are of unknown size and shape. They may exist as free chains or as organized particles and theories to account for the appearance of birefringence may be developed in terms of either form. Another question is the rôle of external forces in producing orientation. The extrusion theory<sup>15</sup> regards the follicle as an organ of extrusion. The continued formation of cells is supposed to generate a pressure which, by forcing the plastic mass through the follicular constriction, deforms the cells and produces orientation at the molecular level by shear. The theory in its simplest terms cannot be wholly true because it would predict that the birefringence should depend on the degree of shear, *i.e.*, on the contraction in area of the follicle. The degree of contraction is very variable whereas the birefringence is more constant for a given class of fibres. Fibrils occur in other cornified tissues<sup>8</sup> in which the effect of a shear cannot be invoked. Thus it is more probable that fibrillation is a spontaneous property of the protein and is to be distinguished from orientation. External forces, on the other hand probably exert a directive influence on the formation of fibrils. Fibrillation itself can be pictured in terms either of free chains or of particles. In the first case the chains may be synthesized in an oriented but hydrated condition in a medium polarized by the flow due to extrusion. A dehydration and condensation occurring at B would lead to the sudden appearance of birefringence. In alternative terms the particles of a corpuscular-type protein can be converted into a fibrous form by end-to-end linkage of the particles. The formation of fibrous structures by this means has been postulated to explain other phenomena, such as the long spacings found by X-rays<sup>16, 17</sup> and electron microscopy in many fibres. Electron micrographs of actin<sup>18</sup>, tropomyosin<sup>19</sup>, and insulin<sup>18</sup> show fibrillated structures most likely formed in this way. Fibrils from wool also show a particulate structure<sup>20</sup>, and long spacings have been found by X-rays<sup>21</sup>. This way of forming fibrils seems therefore to be the more probable. The large-angle features of the X-ray photographs, so characteristic of the keratin-myosin group<sup>22</sup> of proteins, must arise from the pre-formed inner structure of the particles as has already been suggested by ASTBURY in connection with the feather keratin<sup>17</sup> and actin patterns<sup>23, 24</sup>.

The tendency to fibrillate in this manner may be thought to arise from the attraction of end groups or configurations on the opposing faces of the particles.

The fibrous form which arises at the level B and persists throughout the zone BC seems to be held together only by low energy bonds. It is still digestible by enzymes and is converted into the disoriented  $\beta$ -form at moderate temperatures. From the fact that urea is particularly effective in disorienting and dispersing the formation into a non-fibrous condition, it may be concluded that only hydrogen bonds and salt linkages can be holding the structure together. When definite covalent cross links are introduced artificially the fibrous form becomes stable to heating and to urea.

The onset of the *hardening process* is suddenly apparent at the level C. The behaviour of the root when dipped into urea solution is again most informative. The unstabilized lower levels dissolve, but above C the protein only swells and to a diminishing extent as the follicle is ascended, showing the progressive nature of the reaction. Support for the view<sup>1</sup> that a principal reaction of the hardening process is the formation of disulphide cross links between the chains was found in the reversal of the reaction by reduction. Other forms of consolidation are conceivable but have not been demonstrated. Stabilisation seems to involve also the establishment of longitudinal covalent links between the structural elements, leading to a strong formation in the direction of the fibre axis. This is suggested most clearly by the result of heating. The unstabilized zone is disoriented at 90° C *without* contraction in length. The final hair requires a higher temperature and disorientation is accompanied by contraction (supercontraction). The lower zone BC also splits readily at right angles to the axis, above C longitudinal splitting is characteristic. Such observations are most readily explained on the particle theory. The formation of a strong covalent system in the axial direction may distinguish the true fibrous proteins from those proteins, such as actin, which can assume the fibrous form by an end-to-end linkage of particles<sup>18</sup> but are readily dispersed because presumably no secondary reaction of reinforcement occurs. In the keratins the importance of the cystine bridge has been emphasized. However, the occurrence of strong fibres without this form of reinforcement suggests that covalent links may be formed in some other way. ASTBURY has already proposed the "grid-iron transformation" in this connection<sup>23</sup>.

This work was carried out during the tenure of an Imperial Chemical Industries Fellowship. The X-ray tube used was constructed and maintained by Mr J. SIKORSKI to whom the author is greatly indebted. Dr K. M. RUDALL has been generous with advice and criticism.

#### SUMMARY

1. The development of orientation in the presumptive cortex of the follicle of the human hair has been studied by means of birefringence measurements and X-ray photographs.
2. The birefringence rises very rapidly at the constriction of the follicle, and at the same level the typical  $\alpha$ -keratin X-ray diagram appears.
3. The presumptive cortex is divided into the following regions: the isotropic bulb, the fibrous but unconsolidated pre-keratin, the zone of progressive hardening, and the fully hardened hair.
4. The unconsolidated pre-keratin is distinguished by easy digestion by enzymes, dispersion in saturated urea, and disorientation by warming with a fall in birefringence and the appearance of a disoriented  $\beta$ -ray pattern.

#### RÉSUMÉ

1. L'évolution de l'orientation du cortex du follicule du cheveu humain a été étudiée au moyen de mesures de biréfringence et de photographie aux rayons X.

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2. La biréfringence devient rapidement très forte là où le follicule se trouve resserré, en même temps qu'apparaît le diagramme de rayons X caractéristique de la kératine  $\alpha$ .

3. Le cortex se divise en les régions suivantes: le bulbe isotrope, la pré-kératine fibreuse mais encore peu stable, la zone de durcissement progressif et le cheveu dont le durcissement est achevé.

4. La pré-kératine encore peu stable se caractérise par la facilité de sa dégradation par les enzymes, par son aptitude à se disperser dans une solution saturée d'urée, et par la désorientation qu'elle subit lors de son chauffage, désorientation accompagnée d'une diminution de biréfringence et de l'apparition d'un spectre  $\beta$  désorienté.

#### ZUSAMMENFASSUNG

1. Die Entwicklung der Orientierung im Cortex des Follikels des Menschenhaars wurde mit Hilfe von Doppelbrechungsmessungen und Röntgenaufnahmen untersucht.

2. Die Doppelbrechung steigt bei der Einschnürung des Follikels sehr schnell, und in demselben Masse erscheint das typische Röntgendiagramm von  $\alpha$ -Keratin.

3. Der Cortex wird in die folgenden Gebiete eingeteilt: die isotrope Kugel, das faserige, aber nicht konsolidierte Präkeratin, die Zone fortschreitender Härtung, und das vollgehärtete Haar.

4. Das nicht konsolidierte Präkeratin ist gekennzeichnet durch leichte Verdaulichkeit durch Enzyme, Dispersion in gesättigter Harnstofflösung, und Aufhebung der Orientierung durch Erwärmung mit einer Abnahme der Doppelbrechung und dem Auftreten des  $\beta$ -Röntgendiagramms

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## SHRINKAGE OF COLLAGEN

by

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X-ray diffraction patterns have shown that heat shrinkage of native collagen involves conversion from a predominantly crystalline state to an amorphous state (ASTBURY<sup>1</sup>). It has been suggested that this conversion ruptures the links between polypeptide chains, and the chains are then able to fold and assume a more stable configuration (MEYER<sup>21</sup> and BRAYBROOKS, McCANDLISH, AND ATKIN<sup>4</sup>). Thus re-examination and extension of studies on collagen shrinkage should promote understanding of structural changes in proteins of the type involved in denaturation. Moreover, such studies have a direct bearing on certain aspects of some important industries.

## METHODS

*Preparation of tissues*

*Sheepskin.* The skin was placed in a refrigerator at 4° within 1 h of flaying and sampled as required. The wool was shorn close to the surface, and strips, measuring 0.5 cm  $\pm$  0.1 cm by approximately 5 cm, were cut for testing. Samples of the same dimensions were cut for testing the collagen preparations. The mean skin thickness was 0.2 cm and the cross-sectional area was therefore 0.1 cm<sup>2</sup>.

*"Collagen" preparations from sheepskin.* Preliminary tests showed that digestion of certain components of the skin by partial or complete sweating at 25°, or by incubation for 17 h at 25° in 0.4% trypsin (Difco 1:250), 0.4% papain (Parke Davis), or in an aqueous extract containing a mould protease did not affect the shrinkage temperature (s.t.) when heated in water or in the enzyme extracts. However, enzyme digestion alone is insufficient to remove all non-collagenous constituents and other methods were therefore applied. Liming, followed by neutralization with acetic acid, a treatment normally recommended in the preparation of collagen from hide (HIGHBERGER<sup>12</sup>) or from goat skin (THEIS AND JACOBY<sup>24</sup>) lowered the s.t. Thus immersion of skin in saturated Ca(OH)<sub>2</sub> for 17 h at 20°, deliming in dilute acetic acid at pH 4.0 for 2 h and washing in running water for 2 h reduced the s.t. from 67° to 61°.

In view of these findings a drastic procedure was adopted which, though certain to lower the s.t., should remove more of the non-collagenous components than other methods. A pickled pelt from a skin fellmongered by the painting process was used, and the thermostat layer, containing the sebaceous and sudoriferous glands, and the fatty layer were removed from the reticular layer of the dermis in a tannery shaving machine. It was washed in running water for 20 h, neutralized in 0.4% NaHCO<sub>3</sub> for 4 h, washed in running water for 6 h, digested for 18 h in 0.1% trypsin (Difco 1:250) containing

0.5%  $\text{CaCO}_3$  and 0.5% toluene, washed in water for 3 h, dehydrated and degreased twice with ethanol for 2 h, then twice with acetone for 2 h, air-dried, digested with trypsin and dried with ethanol and acetone as before, soaked in benzol for 3 days to remove adhering fat, rinsed twice in acetone, and finally dried in the air. This preparation, termed "Collagen A", had a s.t. of  $55^\circ$ . Removal of traces of Ca by extraction three times in 24 h with 1.0 M  $\text{CH}_3\text{COOH}$  and neutralization by repeatedly adjusting the wash water to  $\text{pH}$  7 yielded "Collagen AI" having a s.t. of  $53^\circ$ .

*Rat tail tendon.* Tendons were dissected from adult rats tails and held at  $4^\circ$  in water under toluene for periods ranging up to 4 days without change in the s.t. Fibres, approximately 0.04 cm in diameter, that is  $0.00126 \text{ cm}^2$  in cross-sectional area, and 3 cm long, were pulled from the tendons for testing.

#### Measurement of shrinkage temperature

Thread was attached to the ends of each strip of skin or collagen, leaving 4 cm between the points of attachment, one end was attached to a wire projecting from the base of a 250 ml beaker containing 200 ml of the liquid to be tested and the other to an aluminium lever which recorded contraction of the strip on the smoked drum of a kymograph. The lever applied a load of 5 g weight to the strip causing slight stretching. After completion of the soaking period the liquid covering the specimen was agitated continuously by a mechanical stirrer and heated to raise its temperature at the rate of  $10^\circ$  per min, and the recording drum was rotated at 1 cm per min. The temperature was marked on the curves at each successive  $5^\circ$  increase and at the s.t. The tendon fibres were tested similarly except that the cotton threads were attached to the fibres at points 2.5 cm apart. Both Collagen A and tendon fibres ruptured when heated in water beyond the s.t.

To test organic compounds available only in small quantities, a micro method was employed which required only 1 ml of solution. A small piece of skin or tendon was attached to the bulb of a thermometer and immersed in the solution to be tested in a test tube, 1 cm internal diameter. The tube was heated in a water bath at the same rate as in the macro method and the temperature noted at which shrinkage became apparent. The results for salicylic acid ( $\text{pH}$  7.0) in Fig. 1, show that the s.t. at zero load was lower than the s.t. at 50 g weight per  $\text{cm}^2$  and the difference increased with concentration. Similar results were obtained using NaCNS solutions. The three values obtained by the micro method were therefore corrected to the corresponding approximate s.t.'s. at 50 g weight per  $\text{cm}^2$  by adding the appropriate differences from Fig. 1. Some compounds, such as *p*-

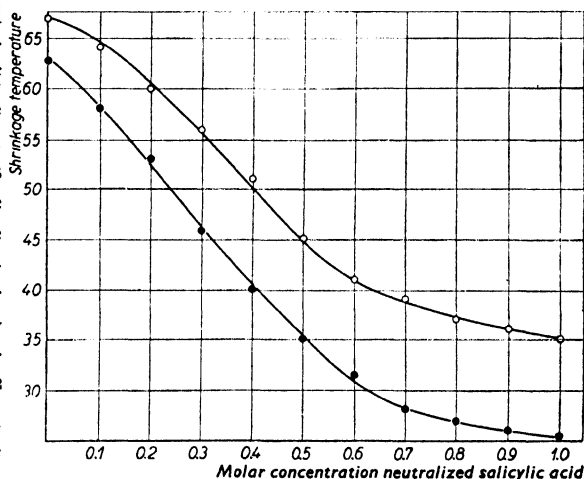


Fig. 1. Effect of concentration of salicylic acid ( $\text{pH}$  7) on the s.t. of sheepskin

o at a load of 50 g weight per  $\text{cm}^2$ -measured by the standard method.

● at zero load-measured by the micro method.

phenylene-diamine and sodium dodecyl sulphate, which were incompletely soluble at room temperature, dissolved on warming at a temperature below the s.t.

#### *Preparation of solutions for testing*

Unless otherwise stated, all the compounds examined were tested at 1.0 M concentration and the solutions were adjusted to  $p_H$  7.0 with HCl or KOH before testing. The glass electrode was used in making all  $p_H$  adjustments and measurements.  $p_H$  7.0 is probably close to the isoelectric point of unlimed collagen (HIGHBERGER<sup>13</sup>; BEEK AND SOOKNE<sup>2</sup>). When the  $p_H$  shifted towards neutrality during the test, as it did in unbuffered solutions in the  $p_H$  range 4 to 10, the values obtained subsequent to testing are reported since they represent more nearly the  $p_H$  of the solution in equilibrium with the tissues during shrinkage.

#### *Time of soaking*

A standard soaking period of 1.5 h before measuring the s.t. was adopted, since this was found to be sufficient for the establishment of a constant s.t. In the highly concentrated solutions used in the elasticity experiments 10 min soaking was sufficient to shrink the tissues.

#### *Elasticity of tissues*

The elasticity of shorn skin and collagen A was measured by cutting strips of these materials of the width and thickness used for s.t. measurement but 7 cm long, attaching threads 0.5 cm from either end, tying one to a fixed wire at the base of a beaker and the other to the hook of a calibrated 500 g spring balance, and applying load by lifting the balance vertically by winding an attached cord on a drum. The length of the strip between the two points of attachment was measured to 0.1 cm for successive loads up to the elastic limit. At zero load the skin was slightly curled and its length could not be accurately measured. Extrapolation of the load-extension curve for several experiments in which the skin was immersed in water showed the length at zero load to be 92.5% of the length at 5 g load. Since these results were reproducible the initial length of each strip could be calculated from the length at 5 g load, thereby obviating the necessity for determining the load-extension curve for each sample.

The elasticity of 0.04 cm diameter tendon fibres was measured similarly except that the length of tendon between the points of attachment was 2.6 cm.

### RESULTS OF SOME PRELIMINARY EXPERIMENTS

#### *Influence of direction on s.t. of skin*

Strips cut from a piece of sheepskin 10 cm square at angles of 0°, 45°, 90° and 135° to one edge all shrank at 67° temperature within  $\pm 0.5^\circ$ . The orientation of the strip in the skin is therefore unimportant.

#### *Thermostat and reticular layers of skin*

Dissection of strips of sheepskin along the fatty layer yielded samples of the thermostat and reticular layers poor and rich, respectively, in collagen bundles. Although both shrank at 67°, the extent of shrinkage of the reticular layer was approximately three times that of the thermostat layer. During shrinkage therefore, whole skin curls with the reticular layer innermost.

Effect of tension applied during the shrinkage

The influence of tension on the s.t. of skin and tendon was measured by applying known loads by the method employed for measuring the elasticity of tissues, but using a more sensitive spring balance calibrated up to a load weight of 12 g. The s.t. was judged by noting the temperature at which the specimen began to contract, movement being detected by sighting it against a millimeter scale. Fig. 2 shows that the s.t. was elevated several degrees by increasing the load from zero to 5 g but little beyond that weight. The curve for tendon fibres continues to rise steeply at loads in excess of 3 g weight. It should be noted that the mean cross-sectional area was only 0.00126 cm<sup>2</sup>, whereas that of the skin was 0.1 cm<sup>2</sup>. Owing to the difficulty of detecting the onset of shrinkage visually, the apparent values under 5 g load are higher than the correct values obtained for the same load from kymograph records. Although the s.t. at zero load for skin, shown in Fig. 2, was 64.5°, most samples of skin tested shrank at 63°.

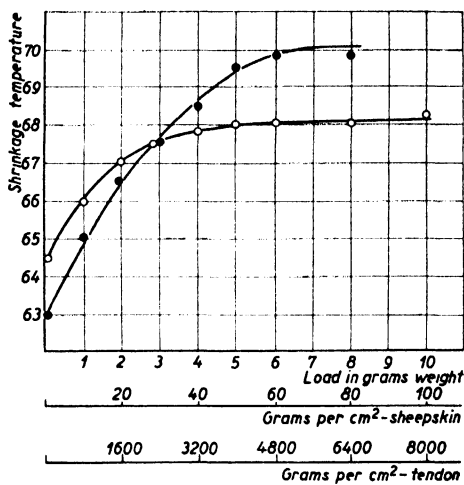


Fig. 2. Effect of load on shrinkage temperature in water  
○ sheepskin ● tendon

Effect of moisture content

Skin was air-dried in one experiment for 3 days, and in another by holding *in vacuo* over H<sub>2</sub>SO<sub>4</sub> for 3 days. Samples were taken at intervals during immersion in water for estimation of moisture content by drying to constant weight at 105° and for measurement of s.t. (Table I). The true moisture contents at the instant of shrinkage would be slightly higher than those reported, particularly after short periods, but this does not obscure the fall in s.t. with increase in moisture content.

TABLE I  
RELATION BETWEEN TIME OF SOAKING DRY SKIN, MOISTURE CONTENT  
AND SHRINKAGE TEMPERATURE (s.t.)

Soaking period (h)	Air-dried skin		Vacuum-dried skin	
	Moisture content (%)	s.t.	Moisture content (%)	s.t.
Nil	11.2	90	12.6	90
0.5	—	73	—	80
1.0	46.2	70	43.4	73
2.3	54.6	67	60.6	70
24.0	72.0	68	74.2	67

A similar fall in the s.t. of collagen A during soaking is also evident from the following figures:

Time of soaking, in h:	nil	0.5	1.0	6.5
S.t.:	65	60	57	55



In spite of this effect no difference was detected between the moisture contents of unshrunk and shrunk samples of collagen A in vapour phase equilibrium with one another.

Elevation of s.t. of tendon fibres by withdrawal of moisture was also demonstrated by heating them in concentrated sucrose solutions. In 1.0 M sucrose the s.t. was elevated to 70°, and in 2.0 M solution to 72°. Similarly in 8 M ethanol the s.t. of tendon was elevated to 70° and in 12 M ethanol to 81°.

#### *Varying the concentration of the shrinkage reagent*

Progressive lowering of s.t. with increase in concentration of several shrinkage reagents is shown in Fig. 3. The curve for KCNS resembles that reported by KÜNTZEL<sup>20</sup>

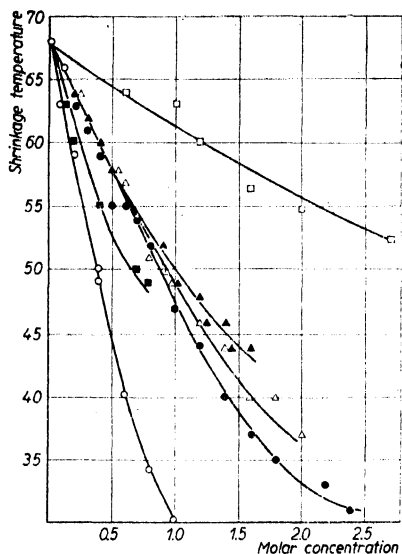


Fig. 3. Effect of concentration on shrinkage temperature, at pH 7

● KCNS      △ NaClO<sub>4</sub>      ▲ KI  
○ salicylic acid,    ■ guanidine,    □ urca

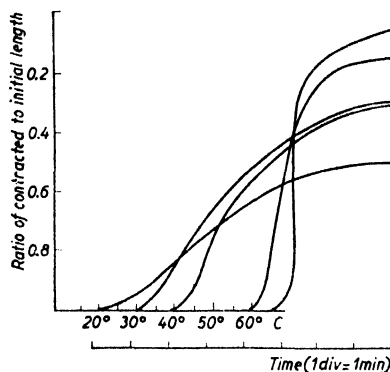


Fig. 4. Reduction in amount of skin shrinkage with increase in concentration of salicylic acid. Reading from left to right along the abscissa, the curves are for the following concentrations of salicylic acid (adjusted to pH 7.0): 1.6 M, 1.0 M, 0.8 M, 0.2 M, nil.

Reduction in the rate and extent of shrinkage with increase in the concentration of salicylic acid (neutralized to pH 7.0) used for soaking at room temperature is evident from Fig. 4, in which several shrinkage curves are superimposed. Partial shrinkage had occurred at room temperature, the amount increasing with the concentration of salicylic acid. Shrinkage was completed by heating in the same solutions.

#### *Shrinkage of skin after soaking in salicylic acid and washing in water*

In Fig. 5 it is shown that when skin was soaked in 1.0 M salicylate for 20 h at 32° and well washed in running water, the s.t. lowering influence of the salicylate was almost entirely removed, but if appreciably shrunk by heating in the salicylate to 37° before washing, shrinkage recommenced at a temperature intermediate between 40°, the value expected if none of the reagent was removed, and 67°, the value expected if removal was complete. Comparison of the salicylate curve with that for skin, partially shrunk in water before washing and reheating, shows that elevation of the temperature at which

shrinkage recommenced was not attributable merely to the change in physical state of the skin, but was apparently due to the use of salicylate to produce this change. Persistent effects of soaking in salicylic acid could be due to residual chemical or to a change in the structure or condition of the collagen.

*Elasticity before and after shrinkage in hot water or in thiocyanate*

As shown in Fig. 6, sheepskin extended to 1.23 times its initial length at a load of 4500 g per cm<sup>2</sup> and contracted to 1.15 times on removing the load. Skin shrunk to 0.42 times its original length by heating in water for 10 min at 70° and cooling showed extension similar to that of unshrunk skin under the same load. However, while under the influence of either heat or concentrated NaCNS the skin displayed long-range elasticity, extending in the NaCNS to more than four times its shrunk length. Skin which was stretched in water at 70° ruptured when the load reached 3000 g per cm<sup>2</sup>.

Similar results were obtained in another experiment in which various strips, some shrunk in hot water and others in CNS<sup>-</sup>, were removed from these liquids before testing the elasticity. Washing one of these strips for 20 h in running water, following complete shrinkage in 10 M KCNS, caused elongation of the strip to about half its original length. Some KCNS may have remained in the tissues, for on heating in water contraction

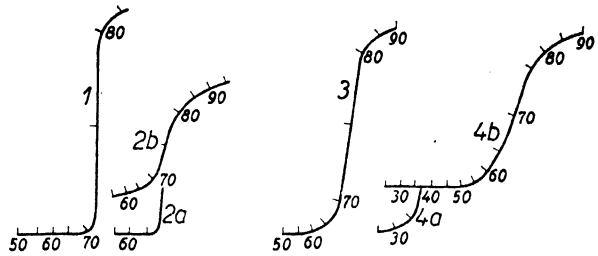


Fig. 5. Resumption of heat shrinkage after partial shrinkage and cooling

Curve 1. Heated in water to 62°, washed for 20 h in water and heated again. (Curve represents contraction during second heating).

Curve 2A. Heated in water to 70°

Curve 2B. Following treatment 2A, sample washed for 20 h in running water and heated in water

Curve 3. Heated in 1.0 M salicylic acid (pH 7.0) to 37°, washed for 20 h in water and heated again. (Curve represents contraction during second heating).

Curve 4A. Heated in 1.0 M salicylic acid (pH 7.0) at 37°

Curve 4B. Following treatment 4A, washed for 20 h in running water and heated in water

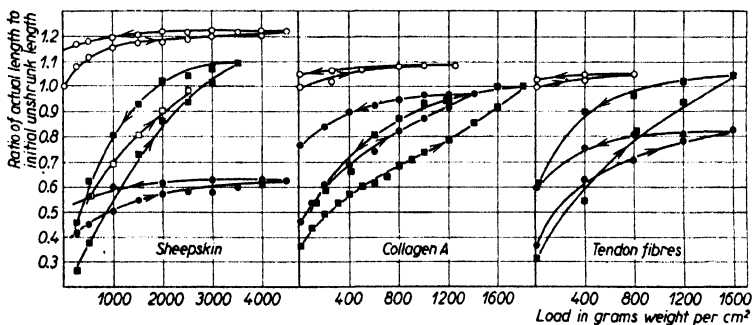


Fig. 6. Elasticity curves for sheepskin, collagen A and tendon fibres

↗ Increasing load      ↘ Decreasing load

- Unshrunk and tested at 18°
- Shrunk and tested in water at 70°
- Shrunk in water at 70° and tested in water at 18°
- Shrunk and tested in 10 M NaCNS at 18°

occurred at 40°, but shrinkage was only half of that strip which was not shrunk in KCNS but which had been washed in water for the same period. By heating a strip to 70° in

water and cooling to room temperature while holding it at its original length, it was set in the extended state.

The curves for collagen A and tendon fibres in Fig. 6 show that, like sheepskin, both tissues displayed restricted elasticity before shrinkage and long-range elasticity in NaCNS. However, they differed from sheepskin in showing greater elasticity when cooled after shrinking in water at 70°. The extension of collagen A after heating and cooling almost equalled its extension in NaCNS solution.

#### THE EFFECTS OF INORGANIC IONS AND $p_H$

##### *Salts containing ions of the lyotropic series*

The s.t. values for the Na and K salts of a variety of inorganic acids tested at  $p_H$  7 and 1.0 M concentration are shown in Table II. The collagen shrinkage activity of these anions agrees with their generally accepted position in the lyotropic series and with their increasing order of hydration.

TABLE II

S.t.'s OF SAMPLES IMMERSSED IN 1.0 M SOLUTIONS OF SALTS CONTAINING ANIONS OF THE LYOTROPIC SERIES, AT  $p_H$  7.0. S.t. IN WATER: SKIN 67°, TENDON 68°

	K salt		Na salt	
	Skin	Tendon	Skin	Tendon
CNS-	47	50	50	52
I-	50°	50°	49	50
ClO <sub>4</sub> -	—**	—**	49	51
NO <sub>3</sub> -	58	65	59	65
Br-	60	66	59	62
NO <sub>2</sub> -	64	64	63	67
Cl-	66	70	65	65
Fe(CN) <sub>6</sub> ----	70	75	—	—
H <sub>2</sub> PO <sub>4</sub> -	72	72	71	75
F-	77	80	72	78
HPO <sub>4</sub> ---*	78	81	77	85
SO <sub>4</sub> ---	(79)**	(76)**	77	83

\* At  $p_H$  7 the phosphate would form a mixture: HPO<sub>4</sub>--- and H<sub>2</sub>PO<sub>4</sub>-

\*\* Owing to low solubility, KClO<sub>4</sub> was not tested and K<sub>2</sub>SO<sub>4</sub> was tested at 0.4 M concentration.

Of the cations tested (Table III), the alkaline earths were all equally effective and, as a class, they exhibited greater activity than the alkali metals. When tested at 0.5 M concentration, that is, at a concentration equivalent to that of the alkali metals, the alkaline earths were again the more effective. Each gave a s.t. of 55°. With the univalent alkali metals however, the shrinkage increased with increase of hydration.

It will be observed that in both the anion and cation series some of the ions raised the s.t. while others lowered it.

Molal solutions of certain oxidizing agents, KClO<sub>3</sub>, KBrO<sub>3</sub> and KIO<sub>3</sub> gave s.t.'s with skin at 64°, 64° and 66°, respectively. Some heavy metal protein precipitants gave the following s.t.'s at the  $p_H$  values indicated: CuSO<sub>4</sub> ( $p_H$  4.0) 58°, FeCl<sub>3</sub> ( $p_H$  1.5) 60°, AgNO<sub>3</sub> ( $p_H$  7.0) 55°, Pb(CH<sub>3</sub>COO)<sub>2</sub> ( $p_H$  7.0) 50°. Some compounds containing N in the anion gave the following values for skin and tendon respectively: sodium nitroprusside Na<sub>2</sub>Fe(CN)<sub>5</sub>NO 40° and 32°, sodium azide NaN<sub>3</sub> 58° and 57°, KCNO 67° and 68°.

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TABLE III

S.t.'s OF SAMPLES IMMERSSED IN 1.0 M SOLUTIONS OF SALTS CONTAINING CATIONS OF THE LYOTROPIC SERIES, AT  $p_H$  7.0. S.t. IN WATER: SKIN  $67^\circ$ , TENDON  $68^\circ$

Cation	Chloride		Sulphate		Relative hydration (WASHBURN AND MILLARD <sup>26</sup> )
	Skin	Tendon	Skin	Tendon	
Ca <sup>++</sup>	50	50	—	—	—
Sr <sup>++</sup>	50	50	—	—	—
Ba <sup>++</sup>	50	50	—	—	—
Mg <sup>++</sup>	52	56	66	70	—
Li <sup>+</sup>	59	61	70	78	14
NH <sub>4</sub> <sup>+</sup>	63	63	73	78	—
Na <sup>+</sup>	66	65	77	83	8.4
K <sup>+</sup>	69	70	(79)*	(76)*	5.4

\* See second footnote to Table II.

Rupture\* of tendon fibres sometimes occurred in salt solutions at  $p_H$  7.0 without prior shrinkage, but in a series of experiments the temperature of rupture was close to the s.t. (usually a degree or two higher), and in some instances it is therefore reported in the tables instead of the s.t. Owing to greater variability in both the s.t. and rupture temperature of tendon fibres less reliance should be placed on these values than on the corresponding values for collagen or skin.

#### Effect of $p_H$

In the absence of salts the s.t. of sheepskin remained constant within the range  $p_H$  4 to 11, but fell sharply outside these limits (Fig. 7). At  $p_H$  13.4 the damage was such that, after contraction, the strip commenced to stretch prior to rupture and the shrinkage curve resembled those for collagen A and tendon. From Fig. 7 it will be noted that not only was the s.t. of the collagen preparations considerably lower than the s.t. of skin but the range of stability of collagen AI to  $p_H$  was narrower.

An experiment was carried out which showed that the use of acid for deliming was primarily responsible for the low s.t. of the collagen preparations used in the present studies, and likewise of those employed by previous workers. Strips of fresh skin were soaked for 19 h in water adjusted to various  $p_H$  values between 1 and 13, the  $p_H$  being readjusted to the initial value after 2 h and 4 h. After washing in water, samples which had been soaked in acid solutions were neutralized in  $KHCO_3$  solutions at  $p_H$  9, and those soaked in alkaline solutions were neutralized in acetate buffer at  $p_H$  5. After 27 h in these solutions, indicators applied to the freshly cut edges showed that the  $p_H$  values

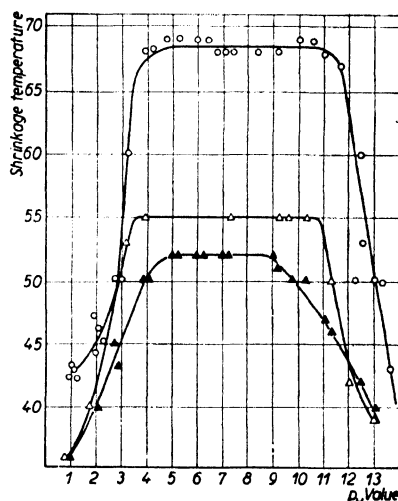


Fig. 7. Effect of  $p_H$  value on shrinkage temperature of sheepskin and skin collagen preparations

○ sheepskin    △ collagen A  
▲ collagen AI

\* When used without qualification in this paper the term "rupture" signifies "rupture of tendon fibre without prior shrinkage".

of all the strips lay within the  $p_H$  range 6 to 8. They were finally washed for 4 h in running water to remove salts. The s.t.'s of these strips, listed in Table IV, show clearly that at  $20^\circ$ , immersion in acid solution at or below  $p_H$  4.0 weakened skin collagen, but alkali, even at  $p_H$  12.8, was without effect and saturated  $\text{Ca}(\text{OH})_2$  elevated it slightly. When heated in solutions of similar alkalinity, however, collagen is severely damaged.

TABLE IV  
EFFECT OF 19 h SOAKING AT  $20^\circ$  IN SOLUTIONS OF VARIOUS  $p_H$   
VALUES ON S.T. OF NEUTRALIZED TISSUES

Reagent	Final $p_H$ value	S.t.	
		Sample A	Sample B
HCl	1.3	52	53
"	2.7	57	56
"	4.0	62	61
KOH	7.2	66	66
"	9.4	66	67
"	10.7	66	66
"	12.8	68	66
$\text{Ca}(\text{OH})_2$	12.8	70	70

#### Varying the $p_H$ of salt solutions

Fig. 8 shows that 0.5 M solutions of  $\text{Na}_2\text{SO}_4$ , KCl, KI,  $\text{NaClO}_4$  and KCN exerted a constant effect on the s.t. at  $p_H$  values between 4 and 11. Their curves are therefore parallel in this range and maintain their relative positions up to  $p_H$  14. In general, however, the relative positions of the curves for all the salts were reversed below  $p_H$  2.5.

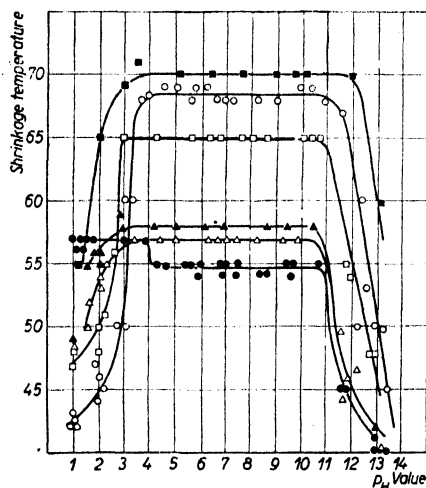


Fig. 8.  $p_H$  curves for sheepskin in the presence of 0.5 M solutions of salts containing various anions

○ No salt    • KCN    △  $\text{NaClO}_4$     ▲ KI  
□ KCl    ■  $\text{Na}_2\text{SO}_4$

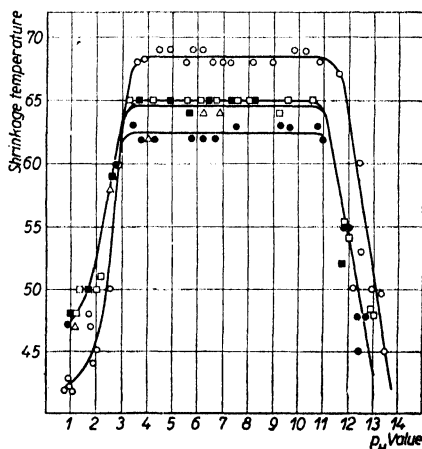


Fig. 9.  $p_H$  curves for sheepskin in the presence of 0.5 M solutions of salts containing various cations

○ No salt    • LiCl    △  $\text{NH}_4\text{Cl}$     ■ NaCl  
□ KCl

The cations all lowered the s.t. above  $p_H$  3.5 and displayed no reversal of lowering below  $p_H$  2.5 (Fig. 9). Owing to serious loss of  $\text{NH}_3$  on heating,  $\text{NH}_4\text{Cl}$  was not tested above  $p_H$  7.

References p. 187.

TABLE V  
S.t.'s OF SKIN AND TENDON, TESTED IN THE SAME SOLUTIONS

Salt at 0.5 M concn	Skin		Tendon		Collagen Al	
	PH 1.0	PH 2.0	PH 1.0	PH 2.0	PH 1.0	PH 2.0
Nil	42	46	38	30	37	42
KCl	47	48	54	52	37	37
KI	49	55	55	56	40	41
NaClO <sub>4</sub>	48	53	53	54	40	40
KCNS	55	55	55	55	40	40

The s.t.'s for tendon were more variable than those for skin, and it was therefore impossible to represent the  $p_H$ -s.t. relationships by smooth curves. From the general trend of the results it was evident that the effect of  $p_H$  and the effect of anions and cations on the s.t. resembled those observed for skin. Reversal of the order of s.t. lowering below  $p_H$  2.5 was not observed, however, and this is confirmed by the results presented in Table V. These values were obtained on the same day with the same solutions under identical conditions. Many of the tendons tested between  $p_H$  1 and  $p_H$  7 both in the presence and absence of salts ruptured without prior shrinkage. By plotting the percentage ruptured against the various  $p_H$  ranges, histograms showing the distribution were obtained (Fig. 10). The mean of the  $p_H$  values, at which rupture occurred in the s.t. experiments to compare various anions, is 4.08 and the standard deviation 1.33  $p_H$  units. The cation figures have a mean of  $p_H$  4.49 and standard deviation of 1.52  $p_H$  units. Examination of the individual shrinkage curves for tendon showed that the extent of shrinkage increased steadily from almost nil at about  $p_H$  6 to a maximum at  $p_H$  9.5. There was also a tendency for the s.t. to rise to a maximum at about this value.

In the absence of salts the percentage of tendons which ruptured in acid solution was much higher. The following figures were obtained by testing 10 fibres at each  $p_H$  value:

$p_H$ . . . . .	1.0	2.0	3.0	3.9	4.6	5.7	6.7
Percentage ruptured before heating . . . . .	nil	80	100	80	30	10	nil
Percentage ruptured before or during heating . . . . .	20	100	100	100	90	30	10

The mean deduced from values for rupture before or during heating is  $p_H$  3.5, and the standard deviation 1.29  $p_H$  units.

The influence of  $p_H$  on the swelling of tendon fibres was studied by determining the ratio of their diameter after immersion in water adjusted to various  $p_H$  values to the initial diameter. The mean of five measurements of diameter along each fibre was obtained with the aid of a microscope and an eyepiece micrometer. The ratios, reported

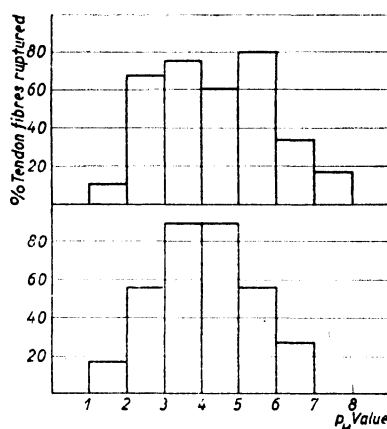


Fig. 10. Histograms showing percentage of tendon fibres ruptured in various  $p_H$  ranges during determination of s.t. Lower curve corresponds to data for anions. Upper curve corresponds to data for cations.

in Fig. 11, reached a maximum at  $p_H$  3.0 in HCl solutions and at  $p_H$  3.6 in oxalic acid solutions. The  $p_H$  values were checked before and after use. Attempts were made also to determine the  $p_H$  of maximum swelling in acetic acid, but the high concentration of acid required produced complete gelatinization at  $p_H$  2.0 and  $p_H$  2.5.

#### THE EFFECT OF ORGANIC IONS

##### *Fatty acids and aliphatic primary amines*

After an initial rise in the s.t. of skin and tendon fibres from  $67^\circ$  to  $72^\circ$  with 1.0 M formic acid and to  $74^\circ$  with 1.0 M acetic acid, a mean reduction of about  $5.5^\circ$  was observed per C atom increment in the length of the chain in the following series of normal fatty acids: acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid, that is up to the  $C_6$  member of the series. Some additional reduction in s.t. probably occurred with lengthening of the chain up to the  $C_9$  member of the series but, owing to the variability of the results, the position of the curve is uncertain (Fig. 12).

A steady fall in s.t. with increase in length of the C chain was also observed in the following series of normal aliphatic primary amines; methylamine, ethylamine, propylamine, butylamine and amylamine (Fig. 12). Unlike the first and second members of the fatty acid series, all the amines reduced the s.t. Moreover, increase in the length of the C chain beyond the  $C_5$  member produced no further appreciable reduction in the s.t., and the mean reduction in s.t., approximately  $3.8^\circ$  per C atom increment in the length of chain, was less than that observed for the fatty acids. It will also be noted that individual amines produced lower s.t.'s than fatty acids having the same length of C chain.

##### *Miscellaneous aliphatic acids, bases and un-ionized compounds*

One of the most striking observations was that all the aliphatic dicarboxylic acids examined, and also citric acid, elevated the s.t. (Table VI). The elevation was more pronounced with tendon fibres than with skin. Among the amines it should be noted that s.t.'s produced by diethylamine and

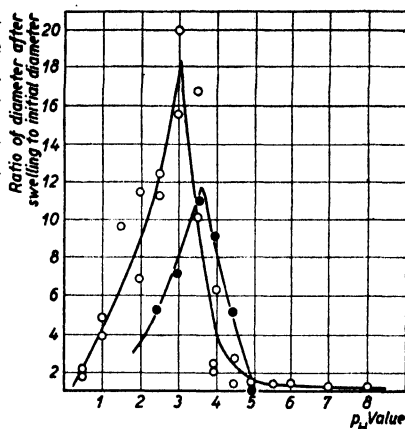


Fig. 11. Swelling of tendon fibres after 15 min at the  $p_H$  values indicated

○  $p_H$  adjusted with HCl  
●  $p_H$  adjusted with oxalic acid

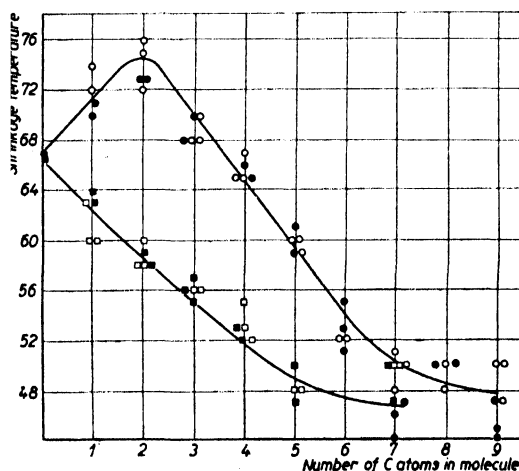


Fig. 12. Progressive lowering of s.t. with increase in length of carbon chain in the fatty acids and aliphatic primary amines, tested at 1.0 M concentration and neutralized to  $p_H$  7

○ fatty acids acting on sheepskin  
● fatty acids acting on tendon fibres  
□ aliphatic primary amines acting on sheepskin  
■ aliphatic primary amines acting on tendon fibres

dipropylamine were close to those produced by the corresponding primary amines, ethylamine and propylamine. The water-soluble un-ionizable organic compounds examined exerted little, if any, effect on the s.t. at 1.0 M concentration. HCHO, which elevated the s.t. very considerably, is a widely used tanning agent.

TABLE VI  
EFFECT OF 1.0 M SOLUTIONS OF SOME ALIPHATIC ACIDS, BASES AND  
UNIONIZED COMPOUNDS, NEUTRALIZED TO pH 7, ON THE S.T. OF  
COLLAGEN. S.t. IN WATER: SKIN 67°, TENDON 68°

Compounds	S.t.	
	Sheepskin	Tendon
<i>Dicarboxylic Acids</i>		
Maleic acid . . . . .	82	82
Fumaric acid . . . . .	80	79
Sebacic acid (pH 8.4) . . . . .	73	75
Adipic acid . . . . .	78	76
Succinic acid . . . . .	76	82
Malonic acid . . . . .	78	83
Oxalic acid . . . . .	78	78
Tartaric acid . . . . .	79	85
<i>Miscellaneous Acids</i>		
Trichloroacetic acid . . . . .	38	36
Hydrogen dodecyl sulphate <sup>*m</sup> . . . . .	47	43
Iodoacetic acid . . . . .	53	51
Bromobutyric acid . . . . .	58	61
Methylamyl sulphosuccinic acid <sup>*</sup> . . . . .	59	58
Bromoacetic acid . . . . .	64	64
Ascorbic acid . . . . .	64	71
Bromopropionic acid . . . . .	67	63
Chloroacetic acid . . . . .	67	67
Gluconic acid . . . . .	68	70
Glycollic acid . . . . .	68	71
Thioglycollic acid . . . . .	69	70
Lactic acid . . . . .	71	68
Citric acid . . . . .	79	80
<i>Secondary Amines</i>		
Dipropylamine . . . . .	55	55
Diethylamine . . . . .	60	60
<i>Miscellaneous Nitrogen Compounds</i>		
Guanidine . . . . .	48	43
Allylamine . . . . .	57	54
Formamide . . . . .	57	60
Triethanolamine (pH 8) . . . . .	58	54
Acetamidine . . . . .	59	59
Ethylene diamine . . . . .	59	62
Thiourea . . . . .	60	57
Malonamide . . . . .	61	58
Urea . . . . .	64	65
Acetamide . . . . .	65	60
Glycine . . . . .	68	74

\* Pure sodium salts of these detergents were used.

<sup>m</sup> Tested by the micro method and corrected.



TABLE VI (continued)

Compound	S.t.	
	Sheepskin	Tendon
<i>Non-ionized Compounds</i>		
2:3 Butane diol . . . . .	63	63
Dioxan . . . . .	65	65
Butyl alcohol . . . . .	65	67
Acetone . . . . .	66	70
Sucrose . . . . .	67	67
Isopropyl alcohol . . . . .	70	67
Ethyl alcohol . . . . .	67	68
Methyl alcohol . . . . .	70	70
Formaldehyde . . . . .	90	85

TABLE VII

EFFECT OF 1.0 M SOLUTIONS OF SOME AROMATIC AND HETEROCYCLIC COMPOUNDS AT pH 7. S.t. IN WATER: SKIN 67°, TENDON 68°

Compound	S.t.	
	Sheepskin	Tendon
Phenylpropionic acid . . . . .	30	20
<i>o</i> -Hydroxy benzoic acid (salicylic acid)	33	—*
Cinnamic acid (pH 8.6) . . . . .	35	20
Gallic acid . . . . .	35	—*
<i>o</i> -Amino benzoic acid (anthranilic acid)	39	35
Dithiosalicylic acid . . . . .	39	36
<i>p</i> -Hydroxybenzoic acid . . . . .	40	30
<i>p</i> -Aminobenzoic acid . . . . .	40	33
Benzoic acid . . . . .	40	33
Phenylpropionic acid . . . . .	40	41
Acetylsalicylic acid . . . . .	41	—*
Benzene sulphonc acid . . . . .	42	33
Benzylamine . . . . .	42	38
<i>o</i> -Toluic acid . . . . .	43	35
<i>m</i> -Phenylene-bis-guanyl guanidine <sup>m</sup> . .	43	43
Phenylacetic acid . . . . .	43	46
Mandelic acid . . . . .	45	45
<i>p</i> -Phenylene diamine . . . . .	46	42
Nicotinic acid . . . . .	52	43
Pyridine . . . . .	52	54
Aniline (pH 4.5) . . . . .	53	52
Piperidine . . . . .	55	53
Benzene disulphonic acid . . . . .	57	60
Pyrogallol . . . . .	62	55
Resorcinol . . . . .	65	61
Isophthalic acid . . . . .	63	65
Naphthalene 1:5 disulphonic acid <sup>m</sup> . .	66	63
Salicylsulphonic acid . . . . .	67	65
<i>o</i> -Phthalic acid . . . . .	67	70

\* The tendon fibres ruptured while attempting to attach them to the lever of the recording apparatus. <sup>m</sup> Tested by the micro-technique and corrected.

#### *Aromatic heterocyclic compounds*

The well-known anionic denaturant, salicylic acid, lowered the s.t. more than did any other compound with the exception of phenyl propionic acid, but it was only slightly more effective than cinnamic acid and gallic acid (Table VII). Benzylamine was the

most effective of the aromatic cationic compounds tested and it is interesting that its s.t. is close to that produced by guanidine, the best of the aliphatic cationic compounds. Most of the aromatic compounds produced s.t.'s below 45°. Least effective were the heterocyclic compounds, the dibasic aromatic acids, and the poorly ionized aromatic compounds. All the acids which produced average s.t.'s below 53° have pK<sub>a</sub> values of less than 5\*. They would therefore be more than 99% ionized at p<sub>H</sub> 7.

#### *Variation in degree of ionization of organic compounds*

By increasing the p<sub>H</sub> of 1.0 M benzylamine in four steps from 4.3 to 9.7 the s.t. was steadily elevated from 37° to 45°, and by increasing the p<sub>H</sub> of 1.0 M aniline in three steps from 4.1 to 6.3 there was a similar elevation of s.t. from 40° to 61°. Since in the absence of salt such changes in p<sub>H</sub> would not affect the s.t., it appears that decrease in the ionization of the bases is responsible for the rise observed. The pK<sub>a</sub> values for benzylamine and aniline are 9.38 and 4.66 respectively at 25°, and they would not differ from these values by more than a fraction of a p<sub>H</sub> unit at the s.t.

#### DISCUSSION

The influence of compounds on the s.t. of collagenous tissues probably depends largely on their degree of adsorption at the collagen-water interface. DOCKING AND HEYMANN<sup>7</sup> have shown that the adsorption of inorganic ions on gelatin conforms with their position in the lyotropic series, and the same order would be expected to hold for their adsorption on the gelatin precursor, collagen. Substances which are strongly adsorbed by gelatin lower the s.t. considerably. If adsorbed only slightly the s.t. may be elevated, particularly if the ion is sufficiently hydrated to withdraw water from the collagen, for drying in air or *in vacuo* produced a considerable rise in s.t. KATZ AND DERKSEN<sup>18</sup> and ASTBURY<sup>1</sup> have shown by X-ray measurements that the side chain spacing in the direction of the salt links increases with hydration from approximately 10 Å to 16 Å for gelatin and to 11.5 Å for collagen. Thus dehydration would bring the adjacent polypeptide chains closer together and increase the opportunity for additional cross linkage between them. Elevation of the s.t. by drying collagen is analogous to the protection against denaturation of globular proteins observed by BEILINSSON<sup>3</sup> when sucrose was incorporated in the denaturant solution.

Amongst the inorganic anions, s.t. lowering varied inversely with hydration. Amongst the fatty acid anions, with the exception of formic acid and acetic acid, s.t. lowering, and presumably adsorption, became more pronounced with increase in the length of the carbon chain. This would be predicted from knowledge that the mutual attraction between organic compounds in solution increases almost linearly with increase in the length of the carbon chain (DUNKEL<sup>8</sup>). The marked influence of many aromatic ions on the s.t. suggests that the possession of a benzene ring is very favourable to adsorption on collagen. Moreover, the introduction of hydrophilic groups, such as OH or NH<sub>2</sub>, into the ring of benzoic acid, and preferably in the ortho position, as in salicylic acid and anthranilic acid, promoted s.t. lowering. The favourable influence of such groups on the adsorption of dyestuffs has led to their classification as "auxochrome"

\* Dissociation constants of most of the compounds tested are published in "LANDOLT-BÖRNSTEIN, *Physikalisch-Chemische Tabellen*" 5th edit., Julius Springer, Berlin. The others were determined by electrometric titration in the author's laboratory.

groups. Presumably they promote the s.t. lowering action of ions also by favouring adsorption. The greater effectiveness of amino- and hydroxy-substituted acids than benzoic acid, and the lesser effectiveness of those containing additional saturated hydrocarbon groups, points to the existence of an optimum polar-apolar balance between the head and the tail of the aromatic ion for maximum s.t. lowering.

A second property which largely determines the effect of a compound on the s.t. of collagen, perhaps by influencing its adsorption, is the extent to which it is ionized at the  $p_H$  of the experiment ( $p_H$  7.0). Compounds, such as the alcohols, which neither ionize nor react chemically with collagen, had little effect on the s.t. at 1.0 M concentration, and compounds such as urea and formamide, which are only feebly ionized at  $p_H$  7, were less effective than well-ionized compounds. But all compounds which lowered the s.t. by  $10^\circ$  or more are completely ionized at  $p_H$  7 and, if anionic in character, they carry only one negative charge. They probably lower the thermal stability of collagen mainly by competitive adsorption at the salt links. Some polyvalent inorganic or organic anions, such as  $SO_4^{-2}$ ,  $PO_4^{-3}$ , citrate, and those liberated by dicarboxylic acids, elevated the s.t., and amongst the aromatic compounds the divalent anions produced less s.t. lowering than the corresponding univalent anions.

Phthalic acid, for example, produced a higher s.t. than benzoic acid. Possession of more than one negative charge may prevent adsorption at the salt link by reducing the polar-apolar balance, or the divalent anions may elevate the s.t. by forming cross links between the negatively charged groups contributed by the lysine and arginine residues in adjacent polypeptide chains. However, if such cross links were formed the s.t. elevation produced by the various divalent aliphatic anions would not be expected to be so alike regardless of the distance separating the two negative charges on the ions. Moreover, DOCKING AND HEYMANN<sup>7</sup> showed that polyvalent anions were only slightly adsorbed on gelatin. Withdrawal of approximately the same amount of water from collagen by the unadsorbed ions, due to the possession of two negatively charged groups which are equally hydrated regardless of the distance between them, is an alternative explanation. Possession of more than one positive charge on the cation did not render it any less effective in lowering the s.t. than the corresponding univalent cation. Thus, *m*-phenylene-bis-guanyl guanidine lowered the s.t. to about the same extent as guanidine, and Ca, Sr and Ba produced lower s.t.'s than Na, K and Li.

A third property which can make an important contribution to the s.t. lowering activity of an ion is resonance. The lowering of s.t. upon introduction of a double or triple bond into the side chain of an aromatic anion, as in cinnamic acid and phenyl propiolic acid, is probably the result of increased resonance in the ion, thereby enhancing its reactivity with hydrogen bonds between the CO and NH groups of adjacent polypeptide chains in collagen. Such ions could lower the s.t. by breaking both salt links and hydrogen bonds. GREENSTEIN<sup>11</sup> attributed the greater denaturing activity of urea and guanidine on egg white than of certain related compounds to their ability to resonate in aqueous solution. The introduction of halogen atoms into the fatty acid anions, as in bromobutyric acid, iodoacetic and especially in trichloroacetic acid, promoted s.t. lowering, yet if the hydrophobic nature of the uncharged portions of the ion were the only consideration the halogenated ions should have lowered the s.t. less than the unsubstituted ions. By attracting electrons within the ion, halogen atoms may increase reactivity with resonating systems in the protein, such as that involving the hydrogen bond. In fact, the hydrogen bond may be the most important site of action for some

ions. However, saturated and unsubstituted ions such as the higher fatty acid anions, would not be expected to react in this way, and for them competitive adsorption at the salt link is probably the only mechanism.

The greater s.t. lowering of anions, in general, than cations may be due to the excess of lysine and arginine residues, as may be seen from published amino acid analyses of collagen and gelatin (ASTBURY<sup>1</sup>) or alternatively, to the number of amide groups, judging by the liberation of ammonia during the conversion of collagen to gelatin and the accompanying fall in isoelectric point from  $p_H$  7.0 or 7.8 for collagen (HIGHBERGER<sup>13</sup>, BEEK AND SOOKNE<sup>2</sup>) to 4.8 for gelatin (HITCHCOCK<sup>14</sup>). An excess of basic groups in collagen would allow penetration of anions into the structure more readily than cations. The explanation of the difference between anions and cations in respect of the influence of their valency on the s.t. will, no doubt, become apparent when more is known of the structure of collagen.

It is difficult to account for the reversal in the relative order of s.t. lowering of skin by inorganic anions with reduction in  $p_H$  value below 2.5. Conversion of the skin collagen to the cationic state may be partly responsible, or there may be association of  $H^+$  ions with the anions being tested within the skin, thereby lowering the local concentration of both ions. Similar reversal in effectiveness of ions with reduction in  $p_H$  value has been demonstrated for other colloidal systems (see, for example, JORDAN LLOYD AND SHORE<sup>17</sup>). Failure to confirm reversal of the lyotropic series when using tendon cannot be explained.

Maximum swelling of tendon fibres and maximum tendency to rupture at about  $p_H$  3.5 suggests that these two effects are related. Both probably depend on the weakening or breaking of entirely different bonds from those which must be broken in order to lower the s.t. It appears that, if the longitudinal strength of the tendon has been weakened by osmotic or other forces, the fibres rupture instead of shrinking when the lateral hydrogen bonds or salt links are broken by heating to the s.t. The greater cross-sectional area and the woven structure of the collagen fibres in the skin specimens would explain their failure to rupture in acid solution.

Long-range elasticity of collagenous tissues was demonstrated in water or in aqueous solution at temperatures above the s.t. The unstabilizing action of 10 M NaCNS on collagen was so pronounced that the s.t. was lowered below 18° and heating was unnecessary. The greater elasticity of the collagen preparation and tendon fibres than skin after heat-shrinking and cooling to 18° suggests that some thermoelastic component was removed from skin during the preparation of collagen, and tendon contains insufficient of such substance to affect its elasticity appreciably. Epidermal keratin was probably the component which restricted the elasticity of skin after cooling.

The influence of ions on physical changes in collagen and gelatin has been the subject of several papers. KATZ AND WEIDINGER<sup>19</sup>, for example, showed that the effect of an anion on the s.t. of collagen depends on its position in the lyotropic series, but the effect of cations was not reported. BRAYBROOKS, MCCANDLISH AND ATKIN<sup>4</sup> and THEIS AND STEINHARDT<sup>23</sup> restricted their s.t. studies on cations to Ca, Mg, Na and K. Similar investigations, but dealing with the relationship between the position of an ion in the lyotropic series and the digestion of collagen in salt solutions, were reported by THOMAS AND FORSTER<sup>25</sup>, and the importance of the series with respect to the swelling, viscosity, setting and precipitation of gelatin was demonstrated by HOFMEISTER<sup>16</sup>, PASCHELES<sup>22</sup>, FREUNDLICH AND SEAL<sup>10</sup> and BÜCHNER<sup>5</sup>. BURK<sup>6</sup> drew attention to the lyotropic series of anions

*References p. 187.*

in relation to the denaturation of globular proteins, as measured by the nitroprusside test for  $-SH$  groups.

Studies of the influence of ions on the excitability of muscle (HÖBER<sup>15</sup>), have shown, as in the present paper, that the effectiveness of inorganic ions is related to their position in the lyotropic series and the action of organic ions depends on their hydrophobic-hydrophilic properties. Moreover, like collagen, myosin is converted by guanidine from a fibrous state to one approaching that of the native globular proteins, as evidenced by reduction in viscosity and in double refraction of flow (EDSALL AND MEHL<sup>9</sup>). The mechanism whereby myosin contracts may, therefore, be similar to that suggested for the shrinkage of collagen.

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#### SUMMARY

The shrinkage temperature (s.t.) of collagenous tissues, such as sheepskin and rat tail tendon, increases with the load applied and with decrease in the moisture content of the tissues. It falls sharply with reduction in  $pH$  value below 4 and with increase beyond 11 and is affected by ions in accordance with their position in the lyotropic series,  $I^-$  and  $CNS^-$  producing the lowest values.

Unionized organic compounds have little influence on the s.t., but the values produced by the cations of primary aliphatic amines, and by the anions of fatty acids higher in the series than acetic acid, decrease with increase in the length of the C chain when tested at  $pH$  7. The aromatic anions of phenylpropionic acid, salicylic acid, cinnamic acid, gallic acid and anthranilic acid lower the s.t. more than any other organic ions tested. Introduction of a second ionized anionic group into an anion represses its s.t. lowering activity, but the introduction of a second ionized cationic group into a cation has no effect.

#### RÉSUMÉ

La température de rétrécissement (shrinkage temperature ou s.t.) des tissus collagènes, comme la peau de mouton et le tendon de la queue du rat, augmente avec l'augmentation de la charge et avec la diminution du degré d'humidité des tissus.

La chute de la température est remarquable, quand la valeur du  $pH$  tombe au-dessous de 4 ou monte au-dessus de 11; elle est influencée en outre par les ions en conformité avec leur position dans la série lyotropique,  $I^-$  et  $CNS^-$  produisant les valeurs les plus basses.

Les combinaisons chimiques non-ionisées n'influencent que légèrement la s.t., tandis que les valeurs produites par les cations des amines aliphatiques primaires et par les anions des acides gras, plus hauts dans la série que l'acide acétique, diminuent avec l'augmentation de la longueur de la chaîne C, quand éprouvées à  $pH$  7. Les anions aromatiques de l'acide phénylpropionique, de l'acide salicylique, de l'acide cinnamique, de l'acide gallique et de l'acide anthranilique réduisent la valeur de la s.t. plus que tous les autres ions organiques éprouvés. L'introduction dans un anion d'un second groupe ionisé d'anions réprime son influence réduisante sur la s.t., tandis que l'introduction dans un cation d'un second groupe ionisé de cations n'a pas d'effet.

#### ZUSAMMENFASSUNG

Die Schrumpfungstemperatur (S.t.) kollagener Gewebe, wie Schafshaut und Rattenschwanzsehne, nimmt mit der angewandten Belastung und mit der Verringerung des Feuchtigkeitsgehaltes

der Gewebe zu. Bei Verminderung des pH-Wertes unter 4 und Erhöhung über 11 fällt sie scharf ab; durch Ionen wird sie in Übereinstimmung mit deren Platz in der lyotropen Reihe beeinflusst, wobei  $I^-$  und  $CNS^-$  die niedrigsten Werte verursachen.

Nicht ionisierte organische Verbindungen beeinflussen die S.t. nur in geringem Masse, aber die Werte, die durch die Kationen primärer aliphatischer Amine und durch die Anionen von Fettsäuren, die in der Reihe höher stehen als Essigsäure, entstehen, nehmen bei Zunahme der Länge der Kohlenstoffkette ab, wenn sie bei pH 7 bestimmt werden. Die aromatischen Anionen von Phenylpropionsäure, Salicylsäure, Zimtsäure, Gallussäure und Anthranilsäure verringern die S.t. mehr als alle anderen untersuchten organischen Ionen. Einführung einer zweiten ionisierten Anionengruppe in ein Anion unterdrückt seine S.t.-erniedrigende Aktivität, während die Einführung einer zweiten ionisierten Kationengruppe in ein Kation keinen Effekt hat.

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# A DISCUSSION OF THE POSSIBILITY OF BANDS OF ENERGY LEVELS IN PROTEINS

## ELECTRONIC INTERACTION IN NON BONDED SYSTEMS

by

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### INTRODUCTION

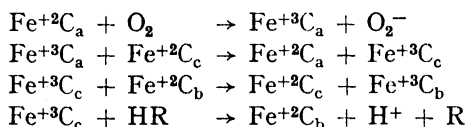
Several recent publications have pointed to the need for a mechanism of protein action which involves not only the action of specific groups and bonds but also the co-operative action of the whole structure.

SZENT-GYÖRGYI<sup>1,2</sup> thinks of a protein as a metallic like structure or a semi-conductor and he and his collaborators have devised experiments on luminescence and photo-conductivity to test this idea. WIRTZ<sup>4</sup> in discussing the transfer of energy and the action of protons on a protein structure invokes bond structures which are characteristic of the protein assembly and not of any particular protein group or chain.

Before discussing possible structures which would confer such a co-operative behaviour on a protein macromolecule, we will review some of the salient facts which seem to us to point to the necessity for a new approach to this question.

### BIOLOGICAL AND OTHER EVIDENCE

The three iron containing proteins cytochrome a, b and c play an important role in the oxidation processes in the animal cell. The cytochromes are bound to the structure of the cell so that they are in effect in an oxidized or reduced state having a tri- or divalent iron atom respectively. The overall reaction can be represented as follows:



Cytochrome a can be oxidized aerobically, and the oxidizing power thus conferred on cytochrome a is transferred through the cytochromes c and b, and the oxidized cytochrome b can then oxidize a reduced compound HR.

It has been assumed<sup>3</sup> that the cytochromes are localized in space at distances very much greater than those normally necessary for intermolecular reaction, which assumption would rule out the possibility of mutual interaction of the cytochromes and leads

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one to seek some agents which can transmit the oxidizing power. There seems to be no evidence for a mechanism based on the action of free radicals or a soluble carrier and indeed the very specific character of the reactions of this system rather precludes such a mechanism.

Another example of the co-operative action of prosthetic groups attached to a common protein appears to be furnished by an observation of P. GEORGE<sup>5</sup> on the reactivity of catalase. This enzyme contains four haem groups (probably arranged in the configuration found by PERUTZ for Haemoglobin). If one of the haems is "blocked" or poisoned by cyanide or azide there is a change in the catalytic reactivity of the remaining haem groups, and it seems therefore, that the activity of the unimpaired enzyme cannot be considered as being made up of the additive reactivity of four individual haems but that there is a special reactivity associated with the assembly of four haems.

The above suggests that there is some special interaction between the prosthetic group and the protein assembly. This interaction is revealed by the change in the oxidation-reduction potential of haemoglobin when the globin is denatured (BARRON<sup>6</sup>).

SPEAKMAN AND ELLIOTT<sup>7</sup> showed that the combination of wool protein with acid dyes is stoichiometric, *i.e.*, the combining power is the same as for simple acids. ASTBURY AND DAWSON and MCARTHUR found that X-ray examination of the dyed proteins showed very little distortion of the protein structure. The setting power of the fibres was, however, greatly impaired showing that the hydrogen ions had, as was indicated by the stoichiometry, penetrated and combined with the protein. These authors concluded that in spite of the inaccessibility of the crystalline protein to the large dye anions, the hydrogen ion penetrates.

The separation of charges envisaged by this mechanism would lead to extremely unstable systems. In the discussion the authors referred to the possibility of electron mobility in proteins as envisaged by HUGGINS<sup>8</sup> and by DENBIGH<sup>9</sup> as a possible solution of the difficulty.

BUCHNER AND KASPERS<sup>10</sup> have shown that when the complex of myoglobin and carbon monoxide is irradiated with light of wavelength of  $280\text{ m}\mu$ , carbon monoxide is liberated, and the ratio of quanta absorbed to moles of carbon monoxide evolved is approximately unity. Myoglobin is a haemprotein in which the haem absorbs at a wave length of  $313$  to  $400\text{ m}\mu$  whereas light of wave length  $280\text{ m}\mu$  is absorbed only by the tyrosine and tryptophane residues of the protein moiety. Nevertheless the absorption of wave length of  $280\text{ m}\mu$  by the protein moiety liberates carbon monoxide from the complex with the same quantum efficiency of unity as does the absorption of wave length  $313$ – $400\text{ m}\mu$  by the haem carbon-monoxide complex, suggesting that the energy absorbed by specific groups in the protein moiety can be transmitted to the reaction bond, *viz.*, the Haem-Fe-Co bond, without a loss of efficiency.

In discussing the action of metal ions and of A. T. P. on the reaction of the protein in muscle, SZENT-GYÖRGYI<sup>11</sup> has pointed out that in effect these reactions appear to occur on a mole fraction basis, that is one or two moles of ions are able to modify the whole behaviour of a mole of protein in spite of the enormous disparity in molal volume. Experience with the reactions of polymers in solution would indicate a volume fraction for the basis of such changes and the only conclusion that can be drawn from SZENT-GYÖRGYI's results is that the protein polymer in these cases is not behaving as an assembly of individual units, that the action is not localized to the site of attack but affects the behaviour throughout the structure.



## ELECTRON BEHAVIOUR IN PROTEIN STRUCTURES

This weight of suggestive but not conclusive experimental evidence has led us to an examination of the possibility of some co-operative valency behaviour in a protein structure which could give rise to the effects described above.

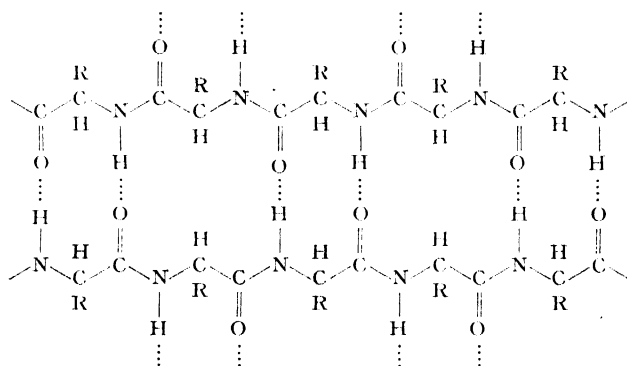
Certain models suggest themselves. Thus in the case of the coupled oxidation reduction of the cytochrome, the protein moiety seems to be behaving as a metal connecting the independent systems as in a multiple oxidation reduction cell.

In the decomposition of the haemprotein -CO complex it appeared that a photon could be transferred through the protein moiety to the site of reaction of the Haem-CO bond. Here the protein appeared to behave as a phosphorescent solid containing colour centres.

Finally the influence of denaturation on the oxidation-reduction potential of a prosthetic group seems to indicate a coupled resonance between the natural protein and the group, a coupling which is modified in the denaturation process.

These points of similarity of common behaviour have led us to seek the possible existence of molecular energy levels in the protein similar perhaps in kind to those existing in a metal or in a polyconjugated structure. The changes in the activity of the prosthetic groups accompanying denaturation would indicate that these molecular energy levels are connected with the particular regular structure in the natural protein.

We ascribe, as has been done before<sup>12, 13, 14</sup> the regular protein structure to the hydrogen bonds between the polypeptide chains thus:



This possibility exists both in the extended  $\beta$  form and in the folded  $\alpha$  form of the chains. Hydrogen bonds can be formed between opposing CO and HN groups of two different polypeptide chains or between these groups in the same folded chain.

We assume therefore that the hydrogen bonding is a characteristic of the structure and enquire what effect this will have on the electron distribution and behaviour of the C, O and N atoms.

The hydrogen bond of the type:



in which the centres are colinear and coplanar, trigonal symmetry has been conferred on the bonds of the nitrogen centre leading to a distortion from the usual pyramidal symmetry of the nitrogen centre. This change in bond symmetry can arise from a

change in the hybridization of the nitrogen. The nitrogen in the hydrogen-bonded structure seems to be entering into bonding not as the structure:

$N (1s)^2 (2s)^2 2p \ 2p \ 2p$  but rather as:

$N (1s)^2 (2s \ 2p)^3 (2p)^2$  in which there is  $sp^2$  hybridization.

Such an electronic structure would lead to the formation of three equivalent electron distributions set at an angle of  $120^\circ$  to each other. The  $(2p)^2$  electron density distribution will be then normal to the plane of the  $> N - H \dots O = C <$  structure and in the same plane as the  $\pi$  electron distribution of the O and C centres of the  $O = C$  group.

Fig. 1 attempts to represent this electron density distribution.

A similar state of affairs arises if we consider a fully ionic N-H bond in this structure. In this case the electronic configuration of the  $N^-$  would be  $(1s)^2 (2s)^2 (2p)^2 \ 2p \ 2p$  and Fig. 2 represents the electron density on the separate centres.

The state represented in diagram 1, probably represents a resonating state between the fully ionic state and that in which the nitrogen has pyramidal symmetry of electron distribution.

In both these cases we seek the energy levels of the molecular orbitals (MO) arising from the separate distributions on the individual centres and these molecular orbitals are occupied by 4 electrons for each unit of  $> NH \dots O = C <$ .

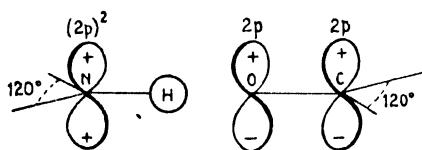


Fig. 1

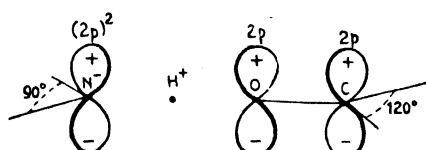
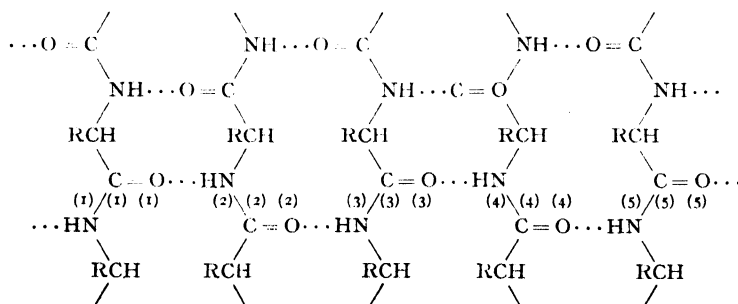


Fig. 2

The importance of the hydrogen bonds is twofold: (a) they give rise to the type of hybridization discussed above and (b) they confer a definite skeleton structure on the configuration, bringing the N and O atoms of neighbouring chains into juxtaposition at a separation of about  $2.65 \text{ \AA}$  and in the same plane. This means that the  $\pi$  electron distributions of the N, O and C atoms are parallel throughout the whole structure.

These are just the conditions for the formation of molecular orbitals from the individual atomic  $\pi$  functions<sup>15</sup> and we propose therefore to explore the system from this point of view.



At each of the centres marked in the above diagram there is an atomic orbital

directed normal to the plane of the structure. The molecular orbitals for the whole linear system will be given by:

$$\psi = \sum_i (a_{Ni} \psi_{Ni} + a_{Ci} \psi_{Ci} + a_{Oi} \psi_{Oi})$$

The secular determinant arising from these  $\psi$  functions will be

$$\begin{vmatrix} q_N - E & \beta_{NC} & 0 & & \\ \beta_{NC} & q_C - E & \beta_{CO} & & \\ 0 & \beta_{CO} & q_O - E & \beta_{ON} & \\ & & \beta_{ON} & q_N - E & \beta_{NC} & 0 \\ & & & \beta_{NC} & q_C - E & \beta_{CO} \\ & & & 0 & \beta_{CO} & q_O - E \\ & & & & \beta_{ON} & q_N - E & \dots \\ & & & & & \vdots & \end{vmatrix} = 0$$

In the equation  $q_i$  represents the integral  $\int \psi_i H \psi_i d\tau$  for the centre  $i$ , and  $\beta$  the integral  $\int \psi_i H \psi_j d\tau$ ;  $\beta$  is zero when  $i$  and  $j$  are not neighbouring centres except in the special case that we have discussed of the centres  $N - H - - - O$  in which case  $\beta$  is given a value a value appropriate to the  $N - O$  distance of  $2.65 \text{ \AA}$ .

For all practical purposes we may consider the determinant an infinite one in which case the solutions are identical with those given by a cyclic structure. Mathematically it is easier to deal with the cyclic determinant, and H. D. URSELL<sup>16</sup> has developed a method to deal with this problem. We have used his method in this work.

We have taken a mean value  $q_m$  as a standard for the integral  $\int \psi_i H \psi_i d\tau$  and expressed individual integrals as  $q_i = q_m + \delta_i$ .

The mean value of  $q_m$  has been obtained from the electron affinities of the individual centres  $NH$ ,  $O =$  and  $C =$  and  $q_m = -13.24 \text{ e.V.}$

The values of  $\delta$  are shown in Table I. The value of  $\beta_{ij}$  has been taken, as is usual, equal to one half the  $\pi\pi$  bond energy in the  $C = O$ ,  $N = C$  and  $N = O$  bonds at the appropriate nuclear separation<sup>17</sup> and these values together with the relevant distances are given in Table I.

TABLE I

$\delta_N = -0.58$	$\delta_O = -1.49$	$\delta_C = 2.07 \text{ eV}$
$\beta_{NO} = -0.20$	$\beta_{CO} = -2.30$	$\beta_{CN} = -1.50 \text{ eV}$
$r_{NO} = 2.65$	$r_{CO} = 1.25$	$r_{CN} = 1.33 \text{ \AA}$

Using these values the solution of the secular equation leads to the result that for such a structure the electron levels are arranged in three bands as shown in Fig. 3; the details are given in Table II.

In the lowest energy state the  $4n$  electrons (where  $n$  is the number of repeat units  $(NH \dots O = C-)$  fully occupy the two lowest bands, each level in each band being occupied by two electrons with opposing spins (See Fig. 3).

It is inherent in the MO method that given a proper orientation of orbitals, there

will be a certain overlap between adjacent orbitals and hence a corresponding lowering in total energy and increase in the mobility of electrons.

TABLE II  
RANGE OF ENERGY BANDS IN eV RELATIVE TO LOWEST  
FILLED LEVEL

	a	b	c
Band 1	0-0.20	0-0.13	0-0.26 doubly filled
Band 2	2.04-2.34	3.17-3.43	2.50-2.89 doubly filled
Band 3	6.57-6.67	6.48-6.60	7.65-7.79 unfilled

a) N pyramidal  
b) N trigonal  
c) NH isoelectronic with O

If the distance between neighbouring centres is too large or if the orientation of the orbitals does not favour overlap we may put  $\beta = 0$ . Let us therefore consider what changes would occur in the properties of the above structure if we put  $\beta_{ON} = 0$ . We should get a set of sharply defined levels, not bands, lying at about the same energy values as the means of the bands in the case discussed above. Each set of 3 levels would correspond to one  $\text{NH} \dots \text{O} = \text{C}$  group in the whole structure. The energy difference between the average energy of one group in the non-localized structure and the energy of the same group of electron orbitals in the localized structure is approximately 0.5-1.0 kcal in the lowest state, in the sense that the non localized is the more stable. For a structure in which there are many such units linked through the H-bonds the total energy of stabilization might be quite considerable and thus an important contribution to the behaviour of such a structure.

The parameters used in the above treatment have been varied; thus an alternative value for the ionization potential of the centre N, 12.24 eV, has been taken corresponding to the change in hybridization<sup>18</sup>.

Another approach is to treat the NH group as isoelectronic with an oxygen centre, and to put  $\beta_{CN} = \beta_{CO}$ . The results based on these alternative values of the parameters are shown in Fig. 3 and Table II. The general results discussed above *viz.*, that the electronic levels are arranged in three bands, is unaltered. The width of the bands and their relative positions are only slightly changed.

If on the basis of an assembly of separate entities  $\text{C} = \text{O} \cdot \text{H} - \text{N}$  the idea of co-operative effect between these structural units is not to be given up entirely, the only way to establish a connection between distant parts of the structure is by means of proton migration reaction, this migration being initiated by some ionogenic reaction, involving the step

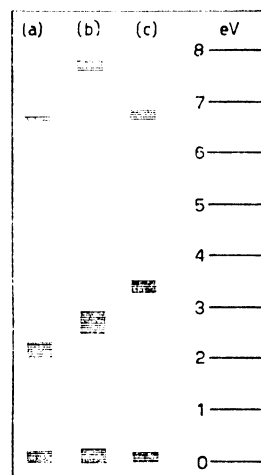
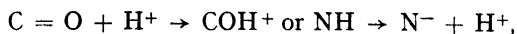
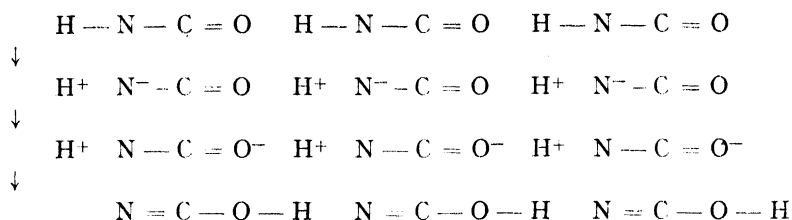
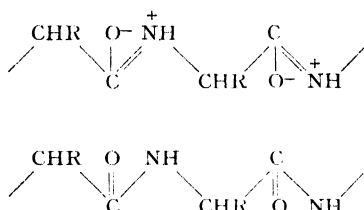


Fig. 3. Position of energy bands in proteins. For details see Table II.

the charge left on the structure after the removal of the H facilitating the migration of the protons. This has been discussed by WIRTZ<sup>4</sup>.



This mechanism does not appear to cover all the biological instances referred to in the introduction, *e.g.*, coupled oxidation reduction, and photon energy transfer. DENBIGH<sup>9</sup> on the other hand does not assume a connection between parallel polypeptide chains but rather a 'conjugation' along a chain which he represents as a resonance between the structures:



DENBIGH made no attempt at a quantitative approach nor does he make it clear that in order to obtain conjugation through a > CHR group one must take into account hyperconjugation involving this group.

It may be of some interest to consider the differences and similarities between metals and such a system as discussed here, particularly because the analogy with metals is being used in biological connection.

1. A protein structure is seen to be non-conducting in the ground state, and can assume conducting properties only on excitation.

2. Judging from the width of the energy bands in a protein structure the electrons are much more localized than in a metal or a graphite structure. The narrowness of these bands suggests that in order to transfer electrons to or from empty or full level of another system the energy levels of the structures must be closely matched. This may indeed have a bearing on specificity of reactions between coupled resonating systems.

3. It follows from (1) that the proposed structure possesses 'semiconductive' properties, but these could never be realized by thermal excitation. The peculiar feature is that the energy gap separating the highest occupied from the lowest unoccupied level is very large compared with  $kT$  and is comparable with bond energies, so that thermal excitation cannot bring about the required electronic transition. In fact the gap seems to be of the order of the energy of the photons which are absorbed in the UV by biological structures.

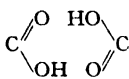
The optical properties of such an array of coupled polypeptide units should be similar to those of the isolated units making up the structure but this work would suggest that the 'coupled' array should manifest slight photoconductivity.

It is worth while to enquire whether other evidence can be adduced to support the principle advanced in this discussion. We have postulated here that there may be interaction between  $\pi$  orbitals belonging to centres which are not connected by a  $\sigma$  bond. These  $\pi$  orbitals may belong to centres in different molecules and the only condition is that the molecules should be oriented by intermolecular forces in such a way that the orbitals on different centres all lie normal to the molecular plane. In the case we have here discussed in detail the H-bond is effective in bringing about the required orientation, and indeed one might expect that in other cases H-bonds would play a major part.

We feel that the observations of SCHEIBE<sup>19, 20, 21, 22</sup> on the absorption spectra of associated cyanine dyes can be interpreted in terms of the above discussion. SCHEIBE found that such aggregates, if irradiated with light polarized in the plane of the molecules, emitted depolarized light. He, therefore, assumed that the absorbed photon was transmitted in the polymer to such points where the curvature of the elongated molecules corresponded to a different direction than that of the polarized light. He further observed that in dried polymers  $\epsilon - \alpha/\beta$  there was a widening of the absorption band with a consequent shift towards the red. SCHEIBE himself suggested some sort of interaction between orbitals of neighbouring molecules. In terms of the method here developed, we can perhaps give a more definite picture of this effect. In Fig. 4 we show how on the basis of the above method the position and the width of the bands would change with increasing interaction between adjacent molecules and the results reveal qualitatively the shift to the longer wave lengths and the widening of the absorption band which SCHEIBE observed. This then is an extension to an oriented but non-bonded array of molecules of the well-known principle in truly conjugated molecules that increasing conjugation increases the wave length and the width of the absorption.

Diamagnetic anisotropy has been observed in molecules in which there is no conjugated ring structure in the accepted sense, as well as in truly conjugated rings<sup>23, 24</sup>. LONDON'S<sup>25</sup> treatment of conjugated ring structures such as benzene naphthalene etc. provides an entirely adequate explanation, based on the quantum mechanical equivalent of a circular current in the plane of the molecules, of the experimental facts for such cases.

If, as we have done here, an interaction between the orbitals of non-bonded but suitably oriented centres is postulated, then a similar treatment could be given for the diamagnetic anisotropy of such molecules as:



the dimer of an aliphatic acid and

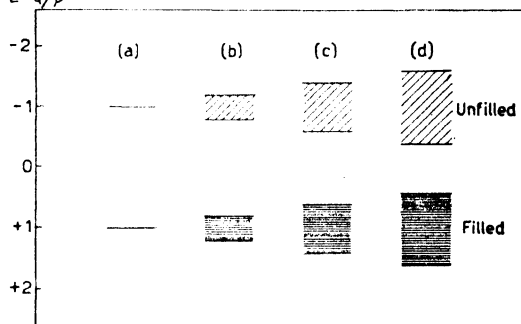
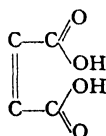


Fig. 4. The influence of the magnitude of the exchange integral  $\beta'$  between adjacent molecules on the spacing and width of the energy bands. The model upon which this diagram is based is that of a linear array of ethylene molecules in which  $\beta$  is the exchange integral between the bonded centres. a)  $\beta' = 0$ ; b)  $\beta' = 0.2\beta$ ; c)  $\beta' = 0.4\beta$ ; d)  $\beta' = 0.6\beta$

In Fig. 5 we show the arrangement of  $\pi$  orbitals which would lead to diamagnetic anisotropy.

In some simplified cases we have calculated, using LONDON's method, very approximate values of the diamagnetic anisotropy arising from the  $\pi$ - $\pi$  interaction between adjacent molecules consisting of identical centres, and with a reasonable choice of parameters have obtained the correct order of magnitude for this effect. We have not given here detailed results because the absolute magnitudes are so approximate. If this concept of the electron behaviour in a protein is correct we should expect an oriented protein structure to show diamagnetic anisotropy, the susceptibility being larger normal to the planes of the hydrogen bonds.

In the case of the protein structure in which the conjugated repeat unit is only of three centres, the separation of the highest filled and the lowest unfilled band is so large that there would be no observable thermal semiconductivity since the requirement for semiconductivity is that this separation should be of the order of 1 eV. We enquire what size of conjugated repeat unit would confer thermal semiconductivity on the structure. Consider a model consisting of an infinite linear array of units each containing  $m$  identical centres connected by  $\sigma$  bonds and having one  $\pi$  orbital orthogonal to the plane of the structure on every centre. Assume that the exchange integral between the unconnected atoms is 1/5 to 1/10 of that between  $\sigma$ -linked atoms. Calculations show that with  $m \sim 12$  the energy separation between the filled and unfilled band is of the required order of magnitude. If the exchange integral between unlinked atoms is bigger than above, the necessary size of the unit for semiconductance is accordingly smaller.

#### SUMMARY

The possibility of conjugation through the orbitals in a protein structure is considered and an attempt has been made to calculate the magnitude of the possible effects.

The results indicate the possibility of a banded electronic structure which confers a small extra stability on the system. The importance of such a model is however that it leads to the possibility of electron 'mobility' and suggests a mechanism for the transfer of electrons, excitation and chemical action through the protein structure.

The model also suggests that an examination of the photoconductivity and diamagnetic anisotropy should reveal the correctness or otherwise of the assumptions on which this work is based.

#### RÉSUMÉ

La possibilité d'une conjugaison par les orbitales dans la structure d'une protéine est envisagée, et une tentative est faite pour calculer l'ordre de grandeur des effets possibles de cette conjugaison.

Les résultats montrent la possibilité de liaisons électroniques conférant une légère stabilité supplémentaire au système. L'importance d'un tel modèle consiste en la possibilité d'une "mobilité" des électrons et suggère un mécanisme pour le transport d'électrons, leur excitation et une action chimique à travers la structure de la protéine. Le modèle suggère également que l'étude de la photoconductivité et de l'anisotropie diamagnétique peuvent permettre d'établir expérimentalement la validité des hypothèses qui sont à la base du présent travail.

#### ZUSAMMENFASSUNG

Die Möglichkeit einer Konjugation durch die Elektronenbahnen in einer Eiweisstruktur wird betrachtet und der Versuch wurde unternommen, die Grösse der möglichen Effekte zu berechnen.

Die Resultate zeigen die Möglichkeit einer Elektronenbandenstruktur an, die dem System eine

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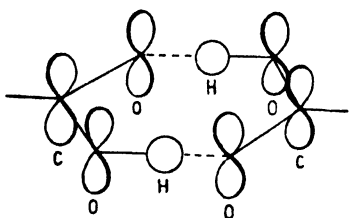


Fig. 5

geringe zusätzliche Stabilität verleiht. Die Bedeutung eines solchen Modells liegt jedoch darin, dass es zu der Möglichkeit der "Beweglichkeit" von Elektronen führt und einen Mechanismus für Elektronenübertragung, Exzitation und chemische Wirkung durch die Eiweisstruktur vorschlägt.

Das Modell führt auch zu der Folgerung, dass eine Untersuchung der Photoleitfähigkeit und der diamagnetischen Anisotropie die Richtigkeit der Annahmen, auf denen das Modell beruht, anzeigen sollte.

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ABSOLUTE REACTION RATE THEORY AND THE  
RESPIRATORY REBOUND\*

by

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Experiments in physical chemistry are generally designed to take advantage of a unique property of a system; thus, those experiments designed to show the presence of unpaired electrons on free radicals must exploit the magnetic properties of these unpaired electrons in order that their existence may be established; as pointed out by SIMON (quoted by PAULING<sup>1</sup>), those techniques designed for a closer and closer approach to the absolute zero of temperature depend upon the utilization of phenomena which appear at these low temperatures in order that they might be achieved. Similarly, a kinetic study of a biological system becomes in the final analysis an exhibition of the kinetic properties unique to a living system. Perhaps the greatest difference between kinetic studies in this type of system and a purely chemical system (of the usual type) is that in the former all reactions proceed in such a fashion as to give rise to the "steady state". This was first noted by HILL<sup>2</sup> and has been defined<sup>3</sup> as existing "... if the rate of change with time of any given parameter, say the concentration of a given reactant of the system, is small compared to the rate at which that reactant is being transformed in one direction. . . ." Its properties may be more clearly demonstrated by a comparison with the properties of a system in equilibrium, which it resembles in superficial aspects only. BURTON<sup>3</sup> has indicated that the differences are:

1. Energy is never dissipated in an equilibrium, but it is dissipated in the steady state.
2. In an equilibrium system forward and back reactions proceed at the same rates; in the steady state there is always a difference in the absolute values of the forward and backward rates.
3. When a system is in complete thermodynamic equilibrium, the decisive factor for the position of the equilibrium is the combined chemical potential energy of the reactants and the products of the system; in a steady state, the "position of the steady state" is determined not only by the potential energy of the particular steady state reaction, but also by a quantity termed the "flux energy" of the steady state.

The existence of a chemical potential factor in the position of the steady state does

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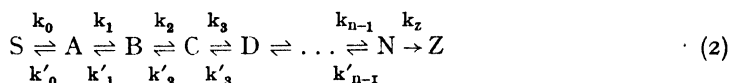
explain why the behaviour of a living system may be influenced by gravitation forces<sup>4</sup>, by electrical forces<sup>5</sup> or by any force which has as its resultant the distortion of the chemically reacting masses bounded by phase produced surfaces. It should also be true that capillary forces might change the position of the steady state, although there seems to be no experimental work extant in this connection, *per se*. This does not include, of course, work with surface active agents, ionic materials, or any demonstration of the effects of osmotic pressure variants on a living system, which certainly partake in their nature of effects on capillarity.

As BURTON<sup>3</sup> has shown, the existence of the "flux rate",  $R$ , serves to characterize further the steady state. In practice, evaluation of this  $R$  quantity provides a convenient means for estimating individual reaction rate constants or concentrations of reactants and products. In the following an attempt will be made to show that in the flux rate we have a quantity of greater theoretical significance than the equations as they stand at present would indicate. The catenary reactions of metabolism have a property peculiar to all reactions of the chain type in that the energies and entropies of the products of one reaction are the energies and entropies for the reactants in the subsequent reaction. In a reaction series of the equilibrated type since there is equality of rates in both directions, and since the universal frequency factor,  $k'T/h$  ( $k'$  is the Boltzmann constant,  $h$ , Planck's constant, and  $T$ , the absolute temperature) which appears in all expression for the reaction rate constant<sup>6</sup> is in identity, there must be a finely balanced relationship between the exponential energy and entropy terms of activation of the forward and reverse reactions; in the steady state, however, there can be no such balance and the conclusion is forced that the flux rate,  $R$ , must actually be determined in part by the difference in the energies and in the entropies of the activated complexes.

The equation given by BURTON<sup>3</sup> for the evaluation of the flux rate is of the form:

$$k_0 S^{\dagger} - k_0' x_1 = k_1 x_1^{n_1} - k_1' x_2^{n_2} = \dots = k_z x_n - k_z' Z = R \quad (1)$$

in which the  $k$ -terms represent the specific reaction constants for the reaction sequence:



in which  $x_1$  is the concentration of  $A$ ,  $x_2$  is the concentration of  $B$ , etc. From the theory of absolute reaction rates<sup>6</sup>, this may be written as:

$$\left\{ e^{-\Delta E_0^{\ddagger}/RT} e^{\Delta S_0^{\ddagger}/R} e^{\Delta n_0^{\ddagger}/R} \right\} S^{\dagger} - \left\{ e^{-\Delta E_1^{\ddagger}/RT} e^{\Delta S_0^{\ddagger}/R} e^{\Delta n_0^{\ddagger}/R} \right\} x_1^{n_1} = \\ \left\{ e^{-\Delta E_1^{\ddagger}/RT} e^{\Delta S_1^{\ddagger}/R} e^{\Delta n_1^{\ddagger}/R} \right\} x_1^{n_1} - \left\{ e^{-\Delta E_2^{\ddagger}/RT} e^{\Delta S_1^{\ddagger}/R} e^{\Delta n_1^{\ddagger}/R} \right\} x_2^{n_2} = \dots \text{etc.} = R \quad (3)$$

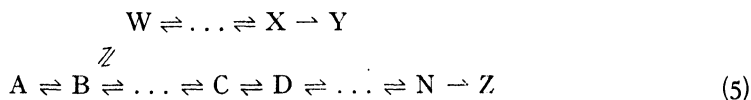
in which  $\Delta E_s^{\ddagger}$  represent the internal energy of the active configuration,  $\Delta S_s^{\ddagger}$ , the entropies of the activated configurations, and the final exponentials represent the increase in the number of molecules involved in the formation of the activated complex. Equation (3) may be simplified. Every concentration appearing in the reaction sequence may be expressed as a function of the concentration of the reactant just immediately preceding it. This statement applies, of course, to only the *simplest* possible steady state reaction sequence, and does not take into consideration the existence of feedbacks, or any other complicating factor<sup>7</sup>. Extension of this substitution leads to:

$$\begin{aligned}
 & \left[ \left\{ e^{-\Delta E_0/RT} e^{\Delta S_0/R} e^{\Delta n_0-I} \right\} - \left\{ e^{-\Delta E'_0/RT} e^{\Delta S'_0/R} e^{\Delta n'_0-I} \right\} l (x_1^{n_1})^{s_1-I} \right] S^{s_1} = \\
 & \left[ \left\{ e^{-\Delta E_1/RT} e^{\Delta S_1/R} e^{\Delta n_1-I} \right\} m (x_1^{n_1})^{s_1-I} - \left\{ e^{-\Delta E'_1/RT} e^{\Delta S'_1/R} e^{\Delta n'_1-I} \right\} n \right. \\
 & \left. (x_2^{n'_2})^{s_1-2} \right] S^{s_1} = \dots R
 \end{aligned} \quad (4)$$

in which every concentration is expressed in terms of the concentration of the primal material,  $S$ , of the reaction sequence, multiplied by a series of factors,  $l$ ,  $m$ ,  $n$ ,  $p$ , etc., to account for the differentiation of numbers of molecules with each successive reaction; introduction of these factors takes into account the fact that it may, for example, require one, two, or three molecules of  $A$  to produce a molecule of  $B$ , or that two or three molecules of  $B$  are produced from the decomposition of one molecule of  $A$ . At first glance it may seem that it is also necessary to introduce another transformation factor from equation to equation, but inspection will show that any factor such as this is already contained in the exponential term, which may be regarded in this instance as a factor of proportionality.

Equation (4) shows that the value of the flux rate,  $R$ , actually depends upon the difference in the exponential terms of the forward and back reactions, upon the concentration of the initial material,  $S$ , and upon a succession of small factors which will have finite values of the order of  $1/3$ ,  $1/2$ ,  $1$ ,  $2$ , or  $3$ . This is equivalent to the statement that the value of the flux rate,  $R$ , depends chiefly upon the value of the difference of the exponential terms to the extent that this difference is equal or nearly in each successive reaction. (In the ideal scheme postulated both for aerobic and anaerobic metabolism it can be seen that the majority of the reactions involve a one-to-one transformation of molecules)<sup>8, 9</sup>. The fact that the last step in the metabolic chain is irreversible leads to a further simplification in the observed flux rate, for the exponential term representing the proportionality factor must be equal to the difference in exponential terms for all previous reactions.

Because of the bifurcation of metabolic pathways permitting the "ideal" biological system to derive energy both aerobically and anaerobically, it is necessary to set up two values of the flux rate for two series of reactions. The bifurcation may be represented as



in which the  $X$  series is the anaerobic series of reactions, the  $A$  series, the aerobic. From (5) it can be seen that a portion of the series is common to both pathways, which fact implies a flexibility in the value of the exponential terms in expression (4). Since there is a series of reactions common to both aerobic and anaerobic metabolism, since it is likely that these two pathways do not of necessity have the same value of the flux rate, and, finally, since in the reactions common to both pathways there is no reason to assume that the nature of the reactants or products are in any way chemically altered, the value of the flux rate must depend primarily upon the differences in the entropies of activation, for it is possible to produce variations in the internal energies of activation only by changing the nature of the materials involved. This point will be discussed later.

The existence of the steady state gives rise to "rebound" or "overshoot" phenomena

in respiration, equivalent in effect to the more familiar oxygen debt of muscle physiology. If a respiring system is suddenly deprived of one of the constituents (in this case, oxygen) necessary for normal metabolism for a short period of time, and then, after this time has elapsed, is returned to an environment where this constituent is present in adequate amounts, it has been observed that for an interval dependent upon the duration of the anaerobic period, respiration will proceed at a far greater rate than it would have under normal conditions. It is this increased rate that is termed "rebound". In the rebound reaction the order of the respiratory reaction has changed, the composite exponential term, the so-called critical increment, has changed, and finally, the rate determining factor or factors in the total reaction sequence appear to have changed temporarily. The change in concentration in the reaction series may be brought about in several ways; first, by actually withholding one of the necessary reactants, second, by suddenly changing the pressure on the reacting series, and third, by suddenly changing the temperature of the system. All three of these methods of inducing rebound have been employed.

BURTON<sup>3</sup> had indicated that overshoot phenomena arise if it is possible to vary the individual values for the specific reaction rate constants of diffusion. He indicated further that the possibility for rebound depends upon an exponential, which, he states, is related to a variation in the potential energy of the system. Because of the properties of the steady state described above, it would not seem that the overshoot depends upon fluctuations in the energies and entropies of activation, and, of these two factors, the variation in entropy of activation is the one which is the most decisive.

It may be postulated that within the first few seconds (or smaller time interval) of the anaerobic period, the aerobic reaction chain will proceed at its normal rate. As the concentration of the next to the last metabolite increase, the back reaction involving its destruction becomes increasingly important. This shift in "importance" of reactions may be transferred back through the chain, because of the accumulation of metabolites, until the entire system has assumed the equilibrium position, or exceeded it slightly in a direction reverse to that of normal aerobic metabolism. When this occurs, and it may occur very rapidly, it may be that it is possible to call into play the normal chain of reactions of anaerobiosis, which must themselves exist in a steady state. The exponential terms of the two steady states must now be equal; in particular, they must be equal to the exponential terms of equilibrium of the aerobic chain, and the value of this new flux rate must be determined by this exponential term. When the switch from aerobiosis to anaerobiosis has been made *within the tissue*, the products of anaerobic metabolism accumulate within the cell or cells until the tissue is again placed in an environment where aerobic metabolism is possible. The anaerobic metabolites may, of course, merely accumulate, or be removed from their locus of production by chemical destruction and transformation, or be removed by means of a physical process, such as diffusion.

A concept such as this may explain why aerobic metabolism is the preferred mechanism when oxygen is present. The pathway of aerobic metabolism beyond that common to both pathways may involve a higher entropy or series of entropies of activation inasmuch as the terminus of the aerobic chain involves several enzymes. The nature of enzymatic reactions seems to be such that a fine degree of spatial organization is necessary for reaction to occur<sup>7</sup>. Such an organization might impose lowered probability in reactions involving these enzymes, manifested by a lowered entropy factor (which enters

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the equations as a positive exponential). The pathway of anaerobic metabolism, on the other hand, might place high energy requirements upon the system.

There is considerable experimental evidence available to support these theoretical conclusions. COLLIP<sup>10</sup> has reported some interesting rebound observations for the tissue *Mya arenaria*. He reported that when this clam was exposed to anaerobic conditions for periods of some days in length, and then returned to fresh, oxygen-containing sea water, the rate of oxygen absorption far exceeded the normal rate; in some cases the normal rate did not appear until after three days. In addition, the rate of oxygen consumption after the anaerobic period varied directly with the temperature. Thus, for example, after four days without oxygen, the rate of absorption was 7.06 ml/100 g drained clam tissue/hour, at 5° C; at 14° C, it was 15.10 ml/100 g drained clam tissue/hour and at 22° C the rate was found to be 21.21 ml/100 g drained clam tissue/hour. The normal rate at 14° C was 1.40 ml/100 g drained tissue/hour. If the tissue was removed from the 14° C environment and placed in an environment at 26° C, the consumption jumped to 11.92 ml/100 g/hour; upon return to the 14° C temperature, the respiration was found to be 7.93 ml/100 g/hour. In these cases the rate of oxygen absorption was determined by the WINKLER method. The data demonstrate the possibility of producing a temperature caused overshoot reaction.

Demonstration of the fact that the overshoot reaction may involve both the end products of aerobic and anaerobic metabolism is found in the work of JATZENKO<sup>11</sup> with *Spaerium corneum*, although he reports temperature fluctuations for his measurements from 14° C to 18° C. He sealed his specimens in a solution of known oxygen concentration (determined by the WINKLER method) for periods of as long as 46 days and then returned them to a solution of high oxygen concentration. He stated that the rate of oxygen consumption was greatly increased and did not return to the normal rate until after 2 days. In these two days and the shell was opened and the siphon was extended. Unfortunately, when a specimen is sealed in a constant environment for periods as long as this, it may be that excreted products appear in the surrounding medium. The effect of these on metabolism is unknown. In spite of this difficulty, and that posed by the fluctuation in temperature, one startling fact is reported by JATZENKO. He found that for the first 14–17 days of anaerobiosis the shells were tightly closed and the animals quiescent in the container. Between the 14th and 17th days, the shells opened up, the siphon was extended and the animals were seen to travel about the vertical walls of the vessel. Simultaneously with this occurrence, the shells and the tissue began to darken in colour. Subsequent analysis showed that this darkening was probably due to the deposition of iron sulphide, both in the shell and in the tissue. On the basis of the theoretical treatment it is suggested that these facts might indicate a switch to an anaerobic type of metabolism *within the tissue*. Such a metabolism must be unique for this tissue inasmuch as it involves the irreversible deposition of iron sulphide.

In this same connection the work of LESSER<sup>12</sup> might be mentioned. He found that the earthworm, *Lumbricus terrestris*, produced fatty acids during an anaerobic period which are not produced in normal oxidative metabolism. When the animals are returned to high oxygen concentration, he found that the oxygen was consumed at an increased rate and that the fatty acids disappeared rapidly. It may be that this, too, can be explained on the basis of the theoretical considerations.

LUND's work<sup>13</sup> on the respiratory overshoot of *Planaria agilis* likewise may fall in line with the theoretical predictions, for he found that the percentage acceleration

of oxygen consumption "is more marked in starved than in fed animals". His measurements were made with the WINKLER titration; there is no statement as to temperature control; the anaerobic period lasted from 8-10 days.

The work of CHOUDHURY<sup>14</sup> on rebound reactions in potatoes (whole) was followed by determining the rate of carbon dioxide production by means of the PETTENKOFER titration. He feels that the existence of an overshoot may be due to one or more causes; "it may be that some oxidizable substance is produced during the anaerobic period and is used up when sufficient oxygen is available, or it may be that some substance is produced in nitrogen which afterwards acts merely as a stimulant and increases the aerobic respiratory rate . . . A third explanation can be that a temporary protoplasmic change . . . is brought about when nitrogen is replaced by air, which leads to an increased availability of sugar for oxidation." The second suggestion, which is based (as CHOUDHURY indicated) on a previous statement of MEYERHOF's<sup>15</sup> might be without validity. The only known method for a material to act as a "stimulant" in a chemical change is for it to function as a catalyst. To be sure, catalysis involves actual participation of the catalysing molecule in the reaction, but, barring poisoning, or other accidents, this catalysis should continue. This is not borne out by the fact that the rebound reaction persists for only a fairly short period of time, and that the rate returns once again to its normal value. The concept of a protoplasmic change (attributed to BLACKMAN<sup>16</sup>) certainly is concerned with a changing entropy value. To attribute this to sugar molecules alone may not be completely justified, on the basis of the previous theoretical treatment.

#### ACKNOWLEDGEMENT

It is with pleasure that acknowledgment is made to Drs. L. J. BERRY and J. L. CRENSHAW for their helpful suggestions and criticisms.

#### SUMMARY

The treatment of BURTON<sup>3</sup> of the respiratory overshoot is reexamined in the light of the absolute reaction rate theory. The value of the flux rate is shown to depend upon the differences in the energies and entropies of activation of the forward and reverse reactions of the individual steps. A mechanism is suggested for the switch within a tissue from aerobic to anaerobic metabolism.

#### RÉSUMÉ

Le traitement de BURTON<sup>3</sup> de l'excès respiratoire est réexaminé à la lumière de la théorie de la vitesse absolue des réactions. On montre que la valeur de la vitesse du flux dépend des différences des énergies et des entropies d'activation des réactions inverses des pas individuels. Un mécanisme est proposé pour le passage à l'intérieur d'un tissu du métabolisme aérobie au mécanisme anaérobie.

#### ZUSAMMENFASSUNG

Der Ausdruck des Atmungsüberschusses von BURTON<sup>3</sup> wird im Lichte der Theorie der absoluten Reaktionsgeschwindigkeit einer erneuten Betrachtung unterzogen. Es wird gezeigt, dass die Grösse der "Flussgeschwindigkeit" (flux-rate) von den Unterschieden in den Aktivierungsenergien und -entropien der entgegengesetzt verlaufenden einzelnen Teilreaktionen abhängt. Es wird ein Mechanismus für den innerhalb der Zelle stattfindenden Umschlag vom aeroben zum anaeroben Stoffwechsel vorgeschlagen.

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CHROMATOGRAPHY OF AMINO ACIDS BELONGING TO  
HOMOLOGOUS SERIES

by

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The positions of the spots produced by various amino acids separated on filter paper according to the chromatographic methods of CONSDON, GORDON, AND MARTIN<sup>1</sup> are determined by their partition coefficients between two phases, one of which is water saturated with organic solvent and the other organic solvent saturated with water. Among the amino acids belonging to a homologous series, these coefficients change in a predictable way with composition and the polarities of their molecules. Addition of OH or COOH groups to the molecule increases polarity and consequently solubility in the water phase, while addition of a CH<sub>2</sub> group to a molecule decreases its polarity and its solubility in water. This note shows the way spot positions, which depend on partition coefficients, vary with composition for amino acids when they are grouped according to composition into the several homologous series to which they can be arranged.

In order to demonstrate the positions and regular distribution of homologues on a chromatogram, mixtures of amino acids falling into different homologous series were analysed on two dimensional chromatograms using collidine saturated with water in one direction and phenol saturated with water containing 0.3 % NH<sub>3</sub> in the direction 90° to the first. This combination of solvents is one used by CONSDON, GORDON, AND MARTIN<sup>1</sup>.

Fig. 1 is a tracing of a two-dimensional chromatogram of a synthetic mixture of amino acids which can be identified by the numbers listed in Table I. The basic aliphatic amino acids, though not strictly homologues, are arranged in a group along a line at the top of the paper. Immediately below this are the dicarboxylic amino acids, aspartic and glutamic acids,  $\alpha$ -amino adipic acid and  $\alpha$ -amino pimilic acid arranged on a smooth curve in order of increasing molecular weight. Close together and below these are the parallel curves formed by the neutral aliphatic branched chain compounds ( $\alpha$ -amino iso-butyric acid, valine and isoleucine) and the straight chain aliphatic neutral amino acids (glycine, alanine,  $\alpha$ -amino normal butyric acid, norleucine and  $\alpha$ -amino heptylic acid). All these appear in order of increasing molecular weight along two smooth curves. The lower curve is identical with that shown before by CONSDON, GORDON, AND MARTIN<sup>1</sup> and by POLSON<sup>2</sup>. The hydroxy amino acids, serine and threonine, fall on a still lower curve that seems to pass through the tyrosine spot. It may be noted that this tyrosine

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TABLE I  
KEY FOR IDENTIFYING AMINO ACIDS IN THE FIGURES

Amino Acid	Number	Amino Acid	Number
Ornithine . . . . .	1	Valine . . . . .	15
Lysine . . . . .	2	Isoleucine . . . . .	16
Arginine . . . . .	3	Serine . . . . .	17
Citrulline . . . . .	4	Threonine . . . . .	18
Aspartic acid . . . . .	5	Phenyl alanine . . . . .	19
Glutamic acid . . . . .	6	Tryptophane . . . . .	20
$\alpha$ -amino adipic acid . . . . .	7	Tyrosine . . . . .	21
$\alpha$ -amino pimilic acid . . . . .	8	Dihydroxy phenyl alanine . . . . .	22
Glycine . . . . .	9	Hydroxy proline . . . . .	23
Alanine . . . . .	10	Proline . . . . .	24
$\alpha$ -amino n-butyric acid . . . . .	11	Histidine . . . . .	25
Norleucine . . . . .	12	Leucine . . . . .	26
$\alpha$ -amino n-heptylic acid . . . . .	13	Methionine . . . . .	27
$\alpha$ -amino iso-butyric acid . . . . .	14		

spot along with those of phenyl alanine and dihydroxy phenyl analine may also be considered to form a group of three substituted phenyl alanines.

As has already been demonstrated, these regularities in positions of spots due to members of a homologous series make partition chromatography well adapted to the discovery and identification of any new amino acids that may exist.

As an illustration of such a use, one can consider results of the analysis<sup>3</sup> already made of the hydrolysates of *E. coli*. A plot of spot positions observed on a typical two-dimensional chromatogram of digests of these organisms appears in Fig. 2. Here spots belonging to homologous series that have been identified with the help of synthetic mixtures of amino acids run simultaneously with the hydrolysate are connected as in Fig. 1. Five spots were consistently observed which could not be identified with amino acids known to occur in nature. These have been designated by the letters a<sub>1</sub>, a<sub>2</sub>, b, c

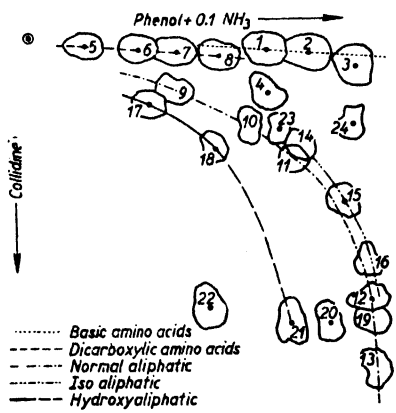


Fig. 1. Tracing of two-dimensional chromatogram of a mixture of amino acids. Spots due to acids of related composition fall on smooth curves.

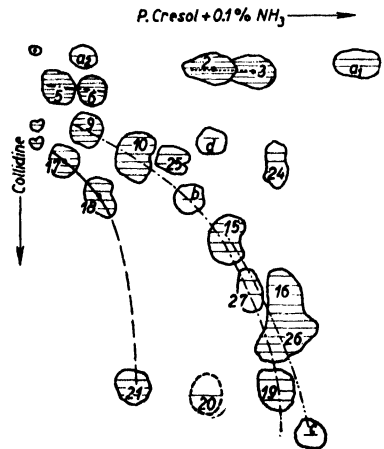


Fig. 2. Tracing of two-dimensional chromatogram of a hydrolysate of *E. coli*. The number of horizontal strips in the spots is indicative of the individual concentrations.

and d. Assuming that they are indeed amino acids some conclusions regarding their probable nature can be drawn from their positions relative to the homologous series lines of Fig. 1. Thus the compounds responsible for spots  $a_1$  and  $a_2$  are probably basic in character. Spot b falls exactly in the position of  $\alpha$ -amino-butyric acid while spot c occupies the position to be expected for a branched chain compound of  $\alpha$ -amino heptylic acid. Spot d does not fall on the line of a homologous series; of the two compounds known to produce spots in approximately this position, one is methionine sulphoxide<sup>4</sup>, the other is citrulline.

Evidently the mere fact that a spot falls on lines joining members of a homologous series is not sufficient for its identification, but the regularities that exist in the partition coefficients of a series is an important aid in the interpretation of two-dimensional chromatograms.

This work has been carried out in the laboratory of DR. RALPH W. G. WYCKOFF, to whom the writer wishes to express his gratitude for many helpful discussions.

#### SUMMARY

Because of the regularities that exist in the solubilities and partition coefficients of amino acids, members of homologous series produce spots in two-dimensional chromatograms of their mixtures which fall on smooth curves. This fact can profitably be used for the analysis of chromatograms of protein hydrolysates and especially as a source of suggestions concerning the character of substances producing unusual spots. Results from analyses of hydrolysates of *E. coli* are cited as an example.

#### RÉSUMÉ

Par suite des relations régulières existant entre la constitution des acides aminés, d'une part, et leur solubilité et leur coefficient de partage, d'autre part, les représentants de séries homologues donnent naissance à des taches, qui, dans des chromatogrammes à deux dimensions, se placent sur des courbes régulières. On peut utiliser ce fait pour l'analyse de chromatogrammes d'hydrolysats de protéines, et en particulier pour avoir une première indication sur la nature d'une substance produisant une tache inattendue. Des résultats obtenus avec des hydrolysats de *E. coli* sont donnés à titre d'exemple.

#### ZUSAMMENFASSUNG

Die regelmässige Abhängigkeit zwischen der Struktur der Aminosäuren einerseits und ihrer Löslichkeit und ihren Verteilungskoeffizienten andererseits bewirkt, dass die Glieder einer homologen Reihe in einem zwei-dimensionalen Chromatogramm regelmässige Kurven bilden. Man kann diese Regelmässigkeit verwerten, um Eiweisshydrolysate zu analysieren, insbesondere können dadurch auch Hinweise auf die Natur einer Substanz, die einen unerwarteten Fleck ergab, erlangt werden. Als Beispiel werden Analysen von Hydrolysaten von *E. coli* angeführt.

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## ON THE FORMATION OF AMINO ACIDS AND PROTEINS IN *TORULA UTILIS* ON NITRATE NUTRITION

by

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In this laboratory it was shown by ROINE<sup>1</sup> that low-nitrogen *Torula utilis* suspended in aerated ammonium sulphate solutions without sugar forms aminodicarboxylic acids, in particular glutamic acid, their amides and alanine from ammonium ions taken up by it. These nitrogen compounds constitute almost the entire soluble N-fraction. In low-nitrogen yeast the fraction of soluble nitrogen vigorously increased during the first 15 minutes when the protein synthesis was very weak. After this the protein synthesis considerably accelerated but still after two hours the soluble fraction contained about twice as much of the nitrogen taken up as the protein. These results speak in favour of the concept that the aminodicarboxylic acids arise as primary amino acids.

VIRTANEN AND CSÁKY<sup>2</sup> noted that low-nitrogen *Torula* suspended in nitrate solution is enriched with the same amino acids as in ammonium salt solution. Qualitatively the only noted difference between the nitrogen compounds formed is the fact that nitrate produces in yeast cells oxime nitrogen which is not produced by ammonium salts.

In quantitative respect the difference between various nitrogen fractions in low-nitrogen *Torula* is great depending on the nature of nitrogen feeding. For instance, the soluble N-fraction increases in nitrate yeast proportionally much less than in ammonium yeast, while the protein synthesis in ratio to the uptake of nitrogen is much more intensive in nitrate yeast than in ammonium yeast. This observation will be dealt with in this paper.

*Torula utilis* yeast was used in the experiments. The strain was the same as used in the previous experiments (ROINE<sup>1</sup>, VIRTANEN AND CSÁKY<sup>2</sup>). The yeast was cultivated in the laboratory in wort agar tube and stored in an ice box to avoid frequent inoculation.

### CULTIVATION OF INOCULATION YEAST

The inoculation yeast required for the cultivation of the actual yeast mass was grown under sterile conditions in four to eight 500 ml boiling flasks, each containing 50 ml nutrient solution of the following composition (solution A):

50 g cane sugar, 3 g  $(\text{NH}_4)_2\text{HPO}_4$ , 4 g  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 g  $\text{K}_2\text{SO}_4$ , 1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{CaCl}_2$ , 1 l tap water.

The pH of the solution was about 6–6.5. There was a heavy precipitate which, however, disappeared with the advance of the growth partly due to the lowering of the pH, partly because the yeast consumed nutrient salts.

The flasks were inoculated with a loopful of yeast from the agar tube. The cultivation occurred in a shaking apparatus in 30° C water thermostat. Period of growth 3–4 days.

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## CULTIVATION OF THE MOTHER YEAST

This phase as well as the subsequent ones was carried out in unsterile conditions. Into a 1.5 l Kluver flask were placed 800–1000 ml of the above solution (solution A) and the total volume of the inoculation yeast (see above). A powerful stream of air was passed through the flask by means of a compressor. The air was purified by a light filter of cotton wool. The flask was kept throughout the course of cultivation in 30° C water thermostat. The pH of the culture solution was kept at 4.5–5.0 by adding 1 N NaOH when needed. The pH was controlled by Lyphan paper. The growth of the yeast mass was followed by taking at certain intervals a 10 ml sample to a centrifuge tube. After 10 min centrifugation (2500 rpm) the yeast was weighed. The cultivation took 10–12 hours. After this the whole yeast mass was separated by centrifugation, washed with tap water, weighed in a tared centrifuge tube and kept over night in an ice box.

## CULTIVATION OF LOW-NITROGEN YEAST

This was also carried out in a Kluver flask into which were measured 1000 ml of the following nutrient solution (solution B):

50 g cane sugar, 3 g  $\text{KH}_2\text{PO}_4$ , 1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{CaCl}_2$ , 1 l tap water.

The pH was about 5, the solution only slightly turbid.

The nitrogen content of the mother yeast obtained in the above manner was reduced by suspending it in the solution B and by aerating it in 30° C water thermostat for 6–7 hours. The pH was regulated as above. The yeast mass increased during this procedure with 1/3–1/2 of the initial fresh weight whereby its N-content simultaneously decreased. Low-nitrogen yeast was separated by centrifugation, washed with tap water and weighed. The mass was stored over night in a centrifuge tube in an ice box. With a longer storage the yeast was suspended in mineral salt solution, free from nitrogen nutrition and carbohydrates of the following composition (solution C):

3 g  $\text{KH}_2\text{PO}_4$ , 1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{CaCl}_2$ , 1 l tap water.

The pH was about 5, the solution only slightly turbid.

## PERFORMANCE OF THE ACTUAL EXPERIMENT WITH LOW-NITROGEN YEAST

Into a Kluver flask were placed 400–450 ml of the above solution C and 200–250 ml of a heavy suspension of low-nitrogen yeast (30–70 g fresh yeast). The flask was aerated as above at 30° C.

After 10 min a 100 ml sample was taken for analysis (0 sample). After 15 min to the suspension was added 100 ml of a solution which contained either 10 g  $(\text{NH}_4)_2\text{SO}_4$  or 15.3 g  $\text{KNO}_3$ , i.e., in either case 2.120 g N. This moment was taken for the start of the experiment. At certain intervals 100 ml samples were taken from the suspension for analysis. All samples were taken into tared centrifuge tubes, the centrifugate was washed with 100 ml of tap water, recentrifuged, and weighed.

When ammonium sulphate served as N-source the pH of the solution was inclined to fall, and therefore 1 N NaOH was added to the solution to maintain its pH at 4.5–5.0. When potassium nitrate formed the N-source the pH had a tendency to rise. This was prevented by adding  $\text{H}_2\text{SO}_4$ .

## ANALYSES

The nitrogen compounds of the yeast were separated into *soluble* and *insoluble fractions* by extracting the soluble substances at a low temperature with 8% trichloroacetic acid (ROINE<sup>1</sup>). The centrifuged, washed and weighed sample of yeast was rinsed with 8% trichloroacetic acid into a measuring cylinder and made up to a definite volume so that the total volume corresponded to 3–4 times the fresh weight of yeast. After shaking the suspension was let to stand over night in an ice box.

**Total nitrogen.** After standing over night an aliquot (2 ml) was taken from the trichloroacetic acid suspension for determination of total nitrogen according to KJELDAHL using 5–6 hours combustion and oxidation with hydrogen peroxide.

**Soluble nitrogen.** The remaining trichloroacetic acid suspension was centrifuged and filtered cell-free through a 3 G 4 Jena glass filter. Nitrogen was determined from an aliquot (2 ml) according to KJELDAHL using 1.5 hours combustion and oxidation with hydrogen peroxide.

**Protein nitrogen** (nitrogen of the insoluble fraction) was calculated as a difference between total nitrogen and soluble nitrogen.

Results which represent the amounts of total, soluble, and protein nitrogen in low-nitrogen yeast fed with ammonia and nitrate nitrogen are given in Tables I and II.

TABLE I

DIFFERENT NITROGEN FRACTIONS OF *Torula* YEASTS FED WITH  $\text{NH}_4\text{-N}$  AND  $\text{NO}_3\text{-N}$  (CALCULATED PER 100 g FRESH YEAST). STRONG AERATION.

Time min	Total nitrogen		Soluble nitrogen		Protein nitrogen	
	mg	increase mg	mg	increase mg	mg	increase mg
NH <sub>4</sub> -experiment						
0	850		113		737	
45	1099	249	301	188	798	61
75	1194	344	366	253	828	91
180	1285	435	361	248	924	187
NO <sub>3</sub> -experiment						
0	816		98		718	
15	850	34	115	17	735	17
75	977	161	159	61	818	100
120	1010	194	154	56	856	136

TABLE II

DIFFERENT NITROGEN FRACTIONS OF *Torula* YEASTS FED WITH  $\text{NH}_4\text{-N}$  AND  $\text{NO}_3\text{-N}$  (CALCULATED PER 100 g FRESH YEAST). WEAKER AERATION THAN IN THE EXPERIMENTS IN TABLE I.

Time min	Total nitrogen		Soluble nitrogen		Protein nitrogen	
	mg	increase mg	mg	increase mg	mg	increase mg
NH <sub>4</sub> -experiment						
0	873		94		779	
45	1121	248	300	206	821	42
75	1167	294	306	212	861	82
195	1279	406	320	226	959	180
NO <sub>3</sub> -experiment						
0	879		95		784	
75	949	70	137	42	812	28
195	1053	174	149	54	904	120

The results recorded in Table I are illustrated by graphs in Fig. 1. The figure shows clearly the essential difference between the increase of soluble nitrogen and protein nitrogen in low-nitrogen yeast with ammonia or nitrate feeding. During 75 minutes ammonia nitrogen produced nearly three times as much soluble nitrogen as protein nitrogen (91 mg or 28.8% protein N of the total uptake 344 mg N) while nitrate nitrogen again produced nearly twice as much protein nitrogen as soluble nitrogen (100 mg or 62.1% protein N of the total uptake 161 mg N). The protein amount formed was thus practically equal in ammonia and in nitrate experiments. The results are quantitatively variable in different experiments, often even considerably, but in all of them the same difference of principle is noticeable.

In one nitrate experiment also amino, amido, ammonia, aspartic acid, glutamic acid, asparagine, glutamine, and alanine nitrogens were determined in addition to soluble and total nitrogen.

Amino N was determined by the Cu-method<sup>3</sup>. It was ascertained with several determinations, that amido nitrogen of glutamine and asparagine does not react in this method.

Amido N was determined according to PUCHER *et al.*<sup>4</sup> as well as ammonia N.

Amido N of glutamine was determined according to SCHWAB<sup>5</sup>.

Amido N of asparagine was calculated by subtracting the amido N of glutamine from total amido N.

Alanine N was determined according to the principle of VIRTANEN *et al.*<sup>6</sup> by the ninhydrin oxidation through determining acetaldehyde in the bisulphite solution according to ROINE AND RAUTANEN<sup>7</sup>.

Aminodicarboxylic acid N was determined according to FOREMAN. The precipitate was dissolved in 1 N acetic acid and the possible nucleotides precipitated with uranyl acetate (ROINE<sup>1</sup>). The nitrogen present in the solution after this was taken for aminodicarboxylic acid nitrogen.

Aspartic acid was determined according to ARHIMO<sup>8</sup> from the solution above. Glutamic acid was calculated by subtracting aspartic acid N from dicarboxylic acid N.

The experiment was performed as follows: 128 g of low-nitrogen yeast were suspended in 1400 ml mineral salt solution (solution C) free from carbohydrate and nitrogen sources, 25 g KNO<sub>3</sub> were added and at certain intervals 250 ml samples were taken from the solution for analysis. Table III gives the results.

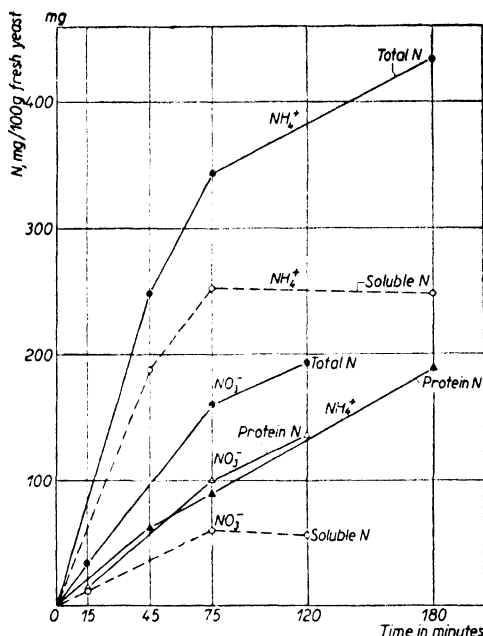


Fig. 1. Low-nitrogen *Torula* suspended in ammonia and nitrate solutions. Different N-fractions during 180 minutes (cf. Table I)

TABLE III

DIFFERENT NITROGEN FRACTIONS IN LOW-NITROGEN YEAST FED WITH NITRATE NITROGEN  
(CALCULATED PER 100 g FRESH YEAST)

Time min	Total N mg	Soluble N mg	Amino N					Amido N			Ammonia N mg
			total mg	dicarboxylic acid, mg	aspartic acid, mg	glutamic acid, mg	alanine mg	total mg	asparagine mg	glutamine mg	
0	714	62.4	39.7	18.92	5.82	13.10	2.53	2.35	1.25	1.10	0.23
15	925	77.3	49.7	25.2	6.23	18.97	5.63	8.6	3.52	5.08	0.58
30	947	92.8	58.0	26.1	6.04	20.06	8.79	12.9	4.58	8.32	1.01
60	1055	123.5	80.0	44.2	6.21	38.00	15.38	7.54	2.16	5.38	1.80
240	1112	118.2	82.8	58.3	6.05	52.25	8.94	5.08	3.22	1.86	0.41

The results recorded in Table III and illustrated graphically in Figs. 2 and 3 show that when low-nitrogen *Torula* takes up nitrate nitrogen the entire increase of soluble nitrogen fraction during the first 15 minutes is practically composed of aminodicarboxylic acids, chiefly of glutamic acid, their amides, alanine, and small amount of ammonia. Besides, some oxime-N is formed which, however, is insignificant in quantitative sense<sup>2</sup>. The small amount of ammonia in the soluble fraction may partly originate from a slight decomposition of glutamine. During the next 45 minutes also other nitrogen compounds are formed to some extent, for the nitrogen of the said amino acids, amides and ammonia then constitute only 77% of the soluble nitrogen. These results are in

good agreement with the findings by ROINE on the uptake of ammonia nitrogen by low-nitrogen *Torula*. The very low amount of soluble nitrogen compared with protein nitrogen in nitrate yeast is illustrated in Fig. 2.

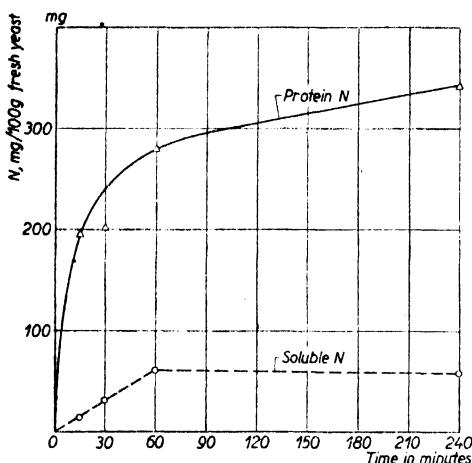


Fig. 2. Low-nitrogen *Torula* suspended in nitrate solution. Protein-N and soluble N during 240 min (cf. Table III)

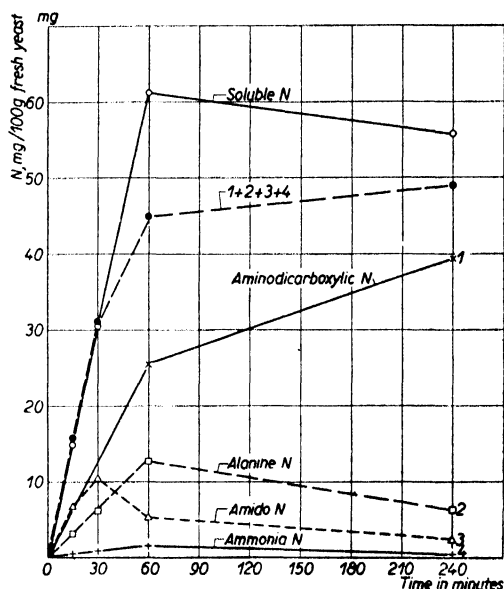
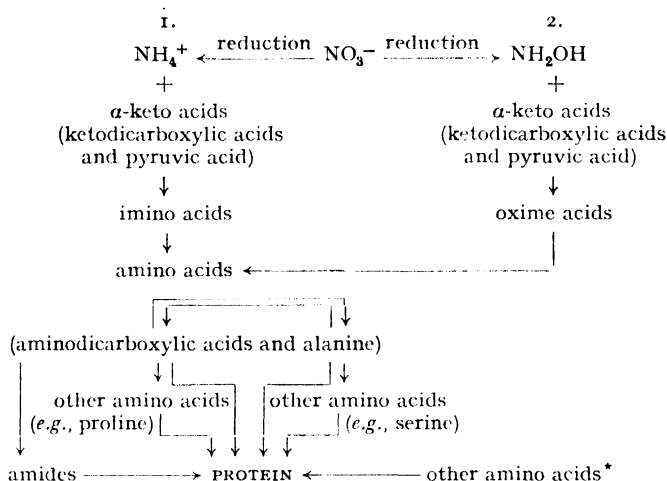


Fig. 3. Composition of soluble N-fraction in *Torula* suspended in nitrate solution (cf. Table III and Fig. 2)

## DISCUSSION

The observations made on low-nitrogen *Torula* indicate that yeast fed either with nitrate or ammonia nitrogen produced the same amino acids and amides which in both cases constitute, during the short experimental time, practically the entire increase in the soluble nitrogen fraction. The increase of this fraction in yeast suspended in ammonium salt solution is much higher than in nitrate solution. Nevertheless, the protein synthesis may reach the same level in both cases. This indicates that 1) the synthesis of aminodicarboxylic acids and alanine takes place more rapidly from ammonia nitrogen than from nitrate nitrogen and that 2) the ample formation of aminodicarboxylic acids and alanine does not as such guarantee an intensive protein synthesis; this depends also on other factors, for instance, possibly on the velocity of the formation of the other amino acids. This with the proviso that when protein is formed all its structure components are present. Whether this holds good, is, however, still unproved. If it does hold good, the results reveal that the synthesis of aminodicarboxylic acids in low-nitrogen yeast takes place with superoptimal velocity in ammonium salt solution, exceeding noticeably the velocity of protein synthesis. In the nitrate solution, again, the synthesis of aminodicarboxylic acids would be approximately optimal corresponding in a high degree to the velocity of protein synthesis. If it is presumed that the synthesis of amino acids occurs according to scheme 1 the accumulation of aminodicarboxylic acids, their amides, and alanine during the short experimental time would be due to the slowness of the synthesis of other amino acids which in turn would retard the protein synthesis.

In the presented scheme besides aminodicarboxylic acids, alanine, too, has been placed to the primary position. It may entirely arise from aminodicarboxylic acids through transamination (VIRTANEN *et al.*<sup>9</sup>, ROINE<sup>1</sup>) but the possibility for its primary formation from pyruvic acid and ammonia is very great since transamination may not be sufficiently rapid to explain alone its formation. Which of the aminodicarboxylic acids, aspartic or glutamic acid, is the primary one has not been dealt with in the scheme.



\* Formed from a source other than aminodicarboxylic acids and alanine.

In the above the complete reduction of nitrate to ammonia has been taken for granted. The rapid formation of oxime from nitrate nitrogen in *Torula* indicates, however, that the hydroxylamine formed in the reduction of nitrate also reacts with the  $> \text{CO}$  group. The maximum amount of oxime N is found according to VIRTANEN AND CSÁKY<sup>2</sup> in *Torula* yeast already 10 min after feeding with nitrate nitrogen. The synthesis of amino acids may, accordingly, take place at least partly over oximes (scheme 2). As far as the synthesis of amino acids proceeds this way to a large extent its different velocity depending on the nitrogen source (ammonia or nitrate) would be easily explicable. The alike velocity of protein synthesis in both cases would even then remain obscure.

### SUMMARY

Low-nitrogen *Torula utilis* takes rapidly up nitrogen in aerated nitrate solution. Both soluble and insoluble nitrogen ("protein nitrogen") are increased in the cells. Soluble nitrogen was extracted from the cells with 8% trichloroacetic acid. The increase of the soluble N-fraction was much smaller than in the corresponding experiment in which *Torula* was fed with ammonia nitrogen instead of nitrate nitrogen. On the other hand, the accumulation of protein in the cells was in many experiments equal in either case. This difference between the influence of nitrate and ammonia nitrogen has been dealt with at a greater length in the Discussion.

In the nitrate experiment the increase of the soluble N-fraction during the first 15 min contained aminodicarboxylic acids, in the first place glutamic acid, their amides, alanine, and some ammonia as well as a very small quantity of oxime nitrogen. These nitrogen compounds correspond roughly to the total soluble N. With a prolonged experimental time the total soluble N was considerably higher than the sum of the nitrogen in the said amino acids and amides. ROINE arrived previously in similar results in regard to the amino acids and amides in soluble N-fraction when examining the uptake of ammonia nitrogen by *Torula* yeast. To alike result led the investigations by RAUTANEN<sup>10</sup> on green plants.

References p. 214.



## RÉSUMÉ

*Torula utilis* pauvre en azote dans une solution de nitrate aérée accumule rapidement de l'azote. La quantité d'azote soluble ainsi que d'azote insoluble (azote protéique) augmente dans les cellules. L'azote soluble a été extrait des cellules avec l'acide trichloracétique à 8%. L'augmentation de la fraction en azote soluble a été beaucoup plus faible que dans une expérience correspondante où *Torula* a été cultivée dans un milieu contenant de l'azote ammoniacal au lieu d'azote nitré. Mais pourtant dans plusieurs expériences l'accumulation de la protéine dans les cellules a été pareille dans les deux cas. Cette différence entre l'influence de l'azote nitré et de l'azote ammoniacal a été traitée plus en détail dans la Discussion.

Dans l'expérience de nitrate la fraction augmentée en azote soluble pendant les 15 premières minutes contenait des acides aminodicarboxyliques, principalement de l'acide glutamique, des amides correspondantes, de l'alanine et un peu d'ammonium ainsi qu'une quantité minime d'azote d'oxime. Ces composés azotés correspondent en gros à l'azote total. Dans le cas où la durée de l'expérience a été prolongée, la quantité d'azote soluble totale a été sensiblement plus élevée que la quantité totale d'azote contenue dans tous ces acides aminés, amides et alanine. En examinant la rétention de l'azote ammoniacal par la levure *Torula* ROINE est arrivé aux mêmes résultats en ce qui concerne les acides aminés et les amides dans la fraction d'azote soluble. Les expériences effectuées avec des plantes vertes par RAUTANEN ont donné les mêmes résultats<sup>10</sup>.

## ZUSAMMENFASSUNG

Die stickstoffarme *Torula utilis* nimmt in durchgelüfteter Nitratlösung schnell Stickstoff auf. Sowohl löslicher als unlöslicher Stickstoff ("Proteinstickstoff") nimmt in den Zellen zu. Die Zunahme der löslichen N-Fraktion, die aus den Zellen mit 8%-iger Trichloressigsäure extrahiert wurde, war viel kleiner als in dem entsprechenden Versuch, in welchem *Torula* in Ammoniumsulfatlösung suspendiert war. Dagegen war die Akkumulation von Protein in den Zellen in vielen Versuchen gleich gross in beiden Fällen. Dieser Unterschied zwischen dem Einfluss des Nitrat- und Ammoniakstickstoffs ist in der Diskussion ausführlicher behandelt worden.

In dem Experiment mit Nitrat enthielt die Zunahme der löslichen N-Fraktion im Lauf der ersten 15 Minuten Aminodicarbonsäuren, an erster Stelle Glutaminsäure, deren Amide, Alanin und auch etwas Ammoniak sowohl wie eine sehr kleine Menge von Oximstickstoff. Diese Stickstoffverbindungen entsprechen im grossen und ganzen dem totalen löslichen Stickstoff. In einer verlängerten Versuchszeit war der lösliche Stickstoff bedeutend höher als die Summe des Stickstoffs in den genannten Aminosäuren, Amidn und Alanin. ROINE ist früher zu ähnlichen Resultaten mit Hinsicht auf die Aminosäuren und Amide in löslicher N-Fraktion gekommen, während er die Aufnahme von Ammoniumstickstoff bei der *Torula*-Hefe untersuchte. Zu diesem Schluss haben desgleichen die Untersuchungen von RAUTANEN<sup>10</sup> mit grünen Pflanzen geführt.

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## THE TOXICITY OF FLUOROACETATE AND THE TRICARBOXYLIC ACID CYCLE\*

by

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The toxic action of the rat poison sodium fluoroacetate presents many features of interest, especially because the molecule is so small and because the C-F bond is so stable (SWARTS<sup>1</sup>) that it cannot combine with -SH groups. BARTLETT AND BARRON<sup>2</sup> have advanced the hypothesis that fluoroacetate is a competitive inhibitor for biochemical reactions in which acetate takes part. Neither they nor others have found any case in which a single enzyme is inhibited by fluoroacetate; but with slices of tissue during the oxidation of pyruvate and other substances, they found that acetate accumulated. In connection with other work upon the paths of oxidation of pyruvate, we have reinvestigated the action of fluoroacetate, in kidney tissue from the guinea pig, and have concluded that the poison has further effects not included in the competitive inhibition hypothesis.

## EXPERIMENTAL

## PREPARATIONS

*Chemicals*

Pure sodium fluoroacetate (NaFlAc) was kindly provided by Dr B. C. SAUNDERS (Cambridge).

Sodium acetate "Analar".

Sodium pyruvate, prepared from pure crystalline pyruvic acid.

Sodium fumarate "B.D.H."

Sodium citrate "Analar".

Sodium malonate prepared from recrystallized malonic acid "KAHLBAUM".

Cozymase was a crude preparation kindly supplied by Dr L. A. STOCKEN, containing about 40 % pure cozymase.

Adenosine triphosphate (ATP) "Boots" was used. Its purity, determined by BAILEY's<sup>3</sup> method was about 90 %.

Oxaloacetic acid was prepared by R. W. WAKELIN from diethyloxaloacetate. Hydrolysis with 4 vol. conc. HCl at -10° C for 2 days. Crystals were filtered and recrystallized from acetone-chloroform mixture.

Oxalosuccinic acid was prepared by R. W. WAKELIN according to OCHOA<sup>4</sup>.

*Enzyme preparations*

As animals, unfasted adult pigeons and guinea pigs fed on ordinary laboratory diet were used. Pigeons were killed by decapitation, guinea pigs by a blow on the head. The *homogenate of pigeon brain* was prepared according to BANGA, OCHOA, AND PETERS<sup>5</sup> and PETERS AND WAKELIN<sup>6</sup>. The *water extract of the acetone powder of pigeon liver* was prepared according to EVANS, VENNESLAND, AND SLOTIN<sup>7</sup>. Ringer phosphate pH 6.5 was used instead of acetate buffer pH 5 (MOULDER, VENNESLAND AND EVANS<sup>8</sup>) in order to avoid any possible influence of acetate on the effects to be observed with

\* Some preliminary statements about this work were presented in the *Dixon Memorial lecture* (June 1948) and in *Proc. Physiol. Soc.*, 26 June 1948.

sodium fluoroacetate.  $\text{MnSO}_4$  (1 mM) and cozymase ( $6 \cdot 10^{-3}$  mM) were added. Liquid phase amounted to 3 ml.

The *kidney brei* (guinea pig) was prepared by removing the kidney quickly and mincing the cortex first with scissors and then with a sharp spatula. From 50 to 200 mg of tissue were used per respirometer bottle. Medium was Ringer phosphate: total volume of fluid was 3 ml; gas phase was  $\text{O}_2$  (80 %).

The *kidney homogenate* (guinea pig) was prepared as follows; the kidneys were quickly removed, cut in two, and cooled in ice according to POTTER<sup>9</sup>. A two minutes' stay in the ice seems to be a maximum and may be even too long as our last experiments seem to indicate. They were trimmed, the medulla discarded, quickly weighed and ground in an ice-cold mortar; 0.9 % KCl (ice-cold) was then added gradually and squeezed through muslin. Grinding in a mortar was deliberately preferred to various homogenizers.

The homogenate containing approx. 150 mg of tissue p. ml was then centrifuged for 30 min at 5000 to 5500 R.P.M. (3500–4700·g) in the cold room and the precipitate suspended in ice-cold 0.9 % KCl buffered with M/10 phosphate pH 7.35.

2 ml containing an amount of tissue corresponding to 150 mg of the kidney cortex were pipetted into respirometer bottles surrounded by ice and already containing  $\text{Mg Cl}_2$  (1.33 mM), ATP (equivalent to 1 mg of the Ba salt p. bottle) 0.2 ml of M/2 phosphate buffer pH 7.35, the inhibitors and sufficient 0.9 % KCl to bring the volume to 3 ml after addition of both enzyme preparation and substrates. The latter were added two minutes after the enzyme preparation.

## METHODS

### Manometric measurements

Unless especially specified, measurements were carried out at 38° C, in Barcroft's differential manometers. Rate of shaking was between 110 and 120 p. min.

### Acetate estimation

LONG's<sup>10</sup> modification of WEIL-MALHERBE's<sup>11, 12</sup> method, based on steam distillation at low pressure and pH about 2, was used. However, phenol red was used as indicator instead of bromothymol blue; the end-point was determined by comparison with a sample of Ringer phosphate pH 7.35 containing a suitable amount of phenol red. 50 ml were collected instead of 55 ml. Recovery, checked each time, was about 80 %.

Deproteinization was carried out with metaphosphoric acid (final conc. 5 %) instead of sulphuric acid.

*Fluoride estimation* was based on the method used by ARMSTRONG<sup>13</sup>. The preparation of the sample in the case of biological material was done by removing the proteins by trichloroacetic acid (final conc. 4 %), neutralizing the filtrate and precipitating the undesirable anions (oxalate, citrate and phosphate) by suitable amounts of  $\text{AgNO}_3$  while maintaining the pH at about 7.0 with NaOH: the solution was centrifuged and the fluoride measured in the supernatant fluid.

### Citrate estimation

PUCHER's *et al.*<sup>14</sup> colorimetric reaction was used after the recommendations of LARDY<sup>15</sup>.

Proteins were precipitated by metaphosphoric acid (final conc. 5 %) according to KREBS AND EGGLESTON<sup>16</sup>. Samples from 0.5 to 3 ml were added to 0.5 ml  $\text{H}_2\text{SO}_4$  50 vol %, made up to 5 ml and reduced to 1.5 to 2.0 ml by boiling. This treatment was omitted when no acetate was used as substrate. Treatment with bromine water was always omitted since no reducing material of vegetable origin was expected. Controls showed that these omissions were justifiable in the cases examined. The final extraction was done with 2 ml  $\text{Na}_2\text{S}$ -dioxane- $\text{H}_2\text{O}$  instead of 10 ml. Readings with the Hilger Spekker photometer (1 cm deep micro cells and Ilford filter 601) gave values of  $d = 0.30$ – $0.35$  for 100  $\mu\text{g}$  citric acid. Standard curves were run simultaneously. Recovery was within the limits of error of the method ( $\pm 0.02$  drum reading).

## CHEMICAL PURITY OF SAMPLES OF SODIUM FLUOROACETATE

We were able to confirm the high degree of purity of the sample of sodium fluoroacetate provided by Dr B. C. SAUNDERS; the fluoride content was 0.05–0.10 %.

As SWARTS<sup>1</sup> already showed, the stability of the C-F bond is such that it is not broken by boiling with conc.  $\text{H}_2\text{SO}_4$  nor by nascent hydrogen.

Sodium fluoroacetate does not react with the -SH group of cysteine (BARTLETT AND BARRON<sup>8</sup>) and does not inhibit the oxidation of pyruvate by the pigeon brain (PETERS AND WAKELIN, 1943, unpublished experiments; this paper), a most sensitive biological test for some -SH poisons. From a chemical point of view, the fluoride atom in a C-F bond seems to be unreactive.

*References p. 229/230.*

Sodium fluoroacetate gives the lanthanum test of KRÜGER AND TSCHIRCH<sup>17</sup> for acetate. Using the amounts and concentrations of reagents recommended by LONG<sup>10</sup>, we however found it approximately 3 times less sensitive to fluoroacetate than to acetate.

In the estimation of acetate by our method when fluoroacetate is present, account must be taken of the "acetic acid" values given by fluoroacetate; 9% of the added amount of fluoroacetate is recovered and titrated as acetic acid. Whether this is due to actual steam distillation of fluoroacetate or to an untimely partial splitting to hydrofluoric and acetic acids has not been determined. It is to be noted that by FRIEDEMANN'S<sup>18</sup> method less than 5% fluoroacetate is recovered. Fluoroacetate is not split by the tissues into fluoride and acetate. Such a phenomenon would have explained both inhibition of the oxygen consumption and the accumulation of acetate. A further proof that the action of fluoroacetate is not due to liberated fluoride is the fact that the residual respiration of a kidney brei was 57.5% inhibited by sodium fluoroacetate (16.6 mM) and only 28% by sodium fluoride (16.6 mM); moreover no traces of freed fluoride could be detected after 2 hours' incubation with the brei; also fluoride inhibits the respiration of brain tissue with pyruvate as substrate, whereas fluoroacetate does not.

## RESULTS

### GENERAL FEATURES OF THE OXIDATION OF FUMARATE AND/OR PYRUVATE BY HOMOGENATES OF KIDNEY CORTEX

It was our aim to study the action of fluoroacetate in a system free from residual respiration and this was achieved, because the homogenates of kidney cortex prepared as indicated are virtually free from oxidizable substrates. They readily oxidize fumarate; pyruvate alone gives only a small oxygen uptake; the addition of pyruvate to fumarate produces an increase of the oxygen uptake over fumarate alone (Fig 1), but this is not comparable with that observed with dialysed brain dispersions (BANGA, OCHOA, AND PETERS<sup>19</sup>). Practically no oxidation occurred in the absence of adenine nucleotides. By using small amounts of fumarate alone, it can be observed that the oxygen taken up accounts for more than the oxidation of fumarate to oxaloacetate (Table I).

As will be seen below, this system is able to synthesize and to oxidize citrate. From experiments with isotopes (BUCHANAN, SAKAMI, GURIN, AND WILSON<sup>20</sup>; WEINHOUSE, MEDES, AND FLOYD<sup>21</sup>; FLOYD, MEDES, AND WEINHOUSE<sup>22</sup>) it is clear that the principal activity of kidney cortex homogenates is to oxidize acetate, acetoacetate and oxaloacetate via the "tricarboxylic" cycle (KREBS AND JOHNSON<sup>23</sup>; KREBS<sup>24</sup>).

In the preparation of this homogenate a somewhat similar system is obtained to that called by GREEN, LOOMIS, AND AUERBACH<sup>25</sup> "cyclophorase"; that is to say it is an enzymatic system oxidizing members of the tricarboxylic cycle via this cycle. For instance it oxidizes citrate well. For these reasons it may be reasonably assumed that the following observations made by us are observations upon the tricarboxylic cycle.

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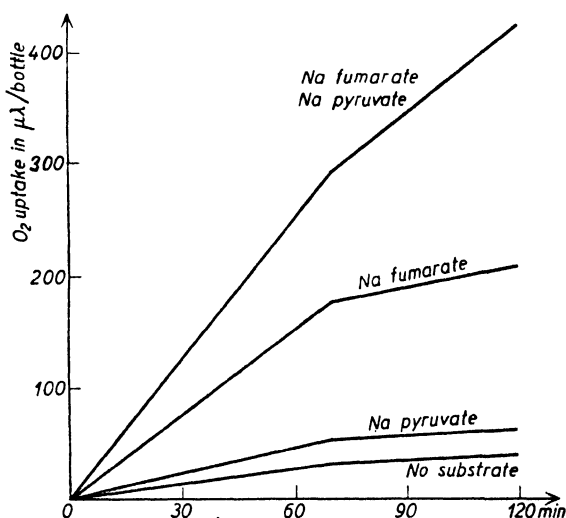


Fig. 1. O<sub>2</sub> uptake by guinea pig kidney homogenate (completed with ATP and Mg<sup>++</sup>). Sodium fumarate: 3.3 mM; Sodium pyruvate: 18.2 mM

TABLE I  
EFFECT OF FUMARATE

Oxygen uptake of centrifuged kidney homogenates with varying amounts of sodium fumarate as substrate (in  $\mu$  moles  $O_2$ /bottle; 2 hours' incubation at  $38^\circ C$ ). All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate, 0.4 mM ATP\*

		Net	Required for oxidation to oxaloacetate
No fumarate . . . . .	0.98	—	—
2 $\mu$ moles fumarate/bottle . . . . .	2.06	1.08	1.0
4 $\mu$ moles fumarate/bottle . . . . .	4.60	3.62	2.0
6 $\mu$ moles fumarate/bottle . . . . .	5.09	4.11	3.0

\* approx. value

SODIUM FLUOROACETATE AS INHIBITOR OF THE OXIDATION OF FUMARATE BY  
HOMOGENATES OF KIDNEY CORTEX

If the only action of sodium fluoroacetate is that of inhibiting utilization of acetate, inhibition of the oxidation of fumarate should be accompanied by acetate accumulation. Our experiments showed that in the presence of fluoroacetate, the oxidation of fumarate, measured by the  $O_2$  consumption, is inhibited, but that this inhibition is not accompanied by an accumulation of acetate (Table II). Hence there must be some other effect of the poison. In the attempt to locate this, other reactions were studied.

TABLE II  
SODIUM FLUOROACETATE AND FUMARATE

Inhibition by sodium fluoroacetate of the oxidation of sodium fumarate (6.6 mM) by centrifuged kidney homogenates, and the effect upon formation of citric and acetic acids. All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate and 0.4 mM ATP.  $O_2$  uptake and acids formed during incubation in air at  $38^\circ C$  for 2 hours

Experiment	NaFlAc (mM)	$O_2$ uptake ( $\mu$ l/bottle)	Inhibition	Acetic acid (mg/bottle)	Citric acid (mg/bottle)
2669	0.0	512	—	None	0.32
	7.5	258	50%	None	0.83
2685	0.0	462	—	0.10	0.18
	3.3	275	40%	0.10	0.37

ATTEMPTS TO STUDY SINGLE REACTIONS OR GROUPS OF REACTIONS INVOLVED IN THE  
TRICARBOXYLIC CYCLE

BARTLETT AND BARRON<sup>2</sup> were not able to find any single enzymatic reaction which shows a sensitivity to fluoroacetate. Particularly interesting is the fact that most of the enzymes involved in the tricarboxylic cycle and subsequent hydrogen transfer were among the enzyme studied: cytochrome oxidase, isocitrate dehydrogenase,  $\alpha$ -keto-glutarate oxidase, succinoxidase, malate dehydrogenase and yeast carboxylase.

Animal pyruvate dehydrogenase can be added to this list.

Table III shows that the oxidation of pyruvate by brain homogenates of the pigeon, is not inhibited by fluoroacetate, whereas it is inhibited by sodium fluoride, a result already obtained by PETERS AND WAKELIN (1943, unpublished experiments).

TABLE III  
PIGEON BRAIN HOMOGENATE  
The effect of sodium fluoroacetate and sodium fluoride upon the oxygen uptake (in  $\mu$ l/bottle) of sodium pyruvate (9.1 mM) in the presence of sodium fumarate (3.3 mM)

Exp.	O <sub>2</sub> uptake (30 min)		
2631 <sup>1, 2</sup>	No inhibitor	426	
	NaF (24 mM)	262	38 % inhibition
	NaFlAc (16.6 mM)	439	No inhibition
2643 <sup>3, 4</sup>	No inhibitor	99	
	NaFlAc (7.5 mM)	96.5	No inhibition

<sup>1</sup> Residual O<sub>2</sub> uptake not subtracted.

<sup>2</sup> Dialysed preparation (4 ½ h), completed with 0.19 mM adenylic acid and 1.33 mM Mg<sup>++</sup>: 220 mg tissue.

<sup>3</sup> Residual O<sub>2</sub> uptake subtracted.

<sup>4</sup> Undialysed preparation: 167 mg tissue.

Since one of the components of the pyruvate oxidase system is pyruvate dehydrogenase, these experiments show that the oxidative decarboxylation of pyruvate is not impaired by fluoroacetate.

In order to obtain some information upon other enzymes of the cycle, *i.e.*, aconitase, oxalosuccinate decarboxylase, fumarase and oxaloacetate decarboxylase, which were not included among the enzymes studied by BARTLETT AND BARRON, the acetone powder of the pigeon liver was used.

This preparation was showed by EVANS *et al.*<sup>7</sup> and MOULDER *et al.*<sup>8</sup> to carry out the following reactions: added fumarate is converted to malate by the fumarase present in this preparation; malate is dehydrogenated to oxaloacetate, this oxidation being coupled with the reduction of pyruvate to lactate; oxaloacetate itself is decarboxylated to pyruvate and carbon dioxide, both spontaneously and enzymatically. The net result is the disappearance of a certain amount of fumarate and its recovery as lactate. Similar reactions occur in the case of added citrate, aconitase, isocitrate dehydrogenase, oxalosuccinate decarboxylase and lactate dehydrogenase being the enzymes involved. Illustration of these processes is summarized in the following scheme: as Fig. 2.

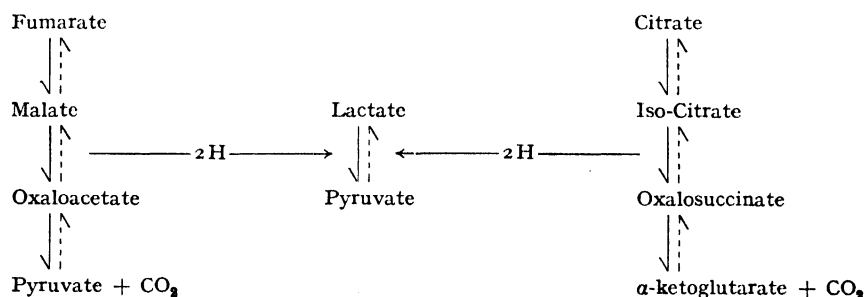


Fig. 2

(All these reactions are reversible, the continuous arrow indicating the predominant direction under the conditions used).

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TABLE IV

DECARBOXYLATION OF OXALOACETATE AND OXALOSUCCINATE BY AN ACETONE POWDER OF PIGEON LIVER  
Substrates provided as sodium fumarate (3.3 mM) and sodium citrate (3.3 mM) respectively. Pyruvate conc.: 20 mM; NaFlAc conc.: 10 mM. Results in  $\mu\text{l CO}_2$ /bottle. Incubated in air for 1 h at 38° C

Experiment	Fumarate as substrate*		Citrate as substrate*	
	Without NaFlAc	With NaFlAc	Without NaFlAc	With NaFlAc
2645	176	175	159	149
2646	168	168	159	150
2647	173	150	158	151

\* Residual subtracted

TABLE V

DECARBOXYLATION OF OXALOACETATE (3.3 mM) AND OXALOSUCCINATE (3.3 mM) BY AN ACETONE POWDER OF PIGEON LIVER

Substrates tipped into the main compartment from KEILIN's tubes at  $t_0$ . Temp. 28° C. NaFlAc 10 mM; Na malonate 25 mM; no pyruvate, no cozymase ( $\mu\text{l CO}_2$ /bottle)

Experiment	Substrate	Spontaneous (boiled enzyme)	Control	NaFlAc	Malonate
2706	oxaloacetate	115*	189*	194*	122*
2707	oxalosuccinate	110**	159**	183**	167**

\* 30 min incubation

\*\* 10 min incubation

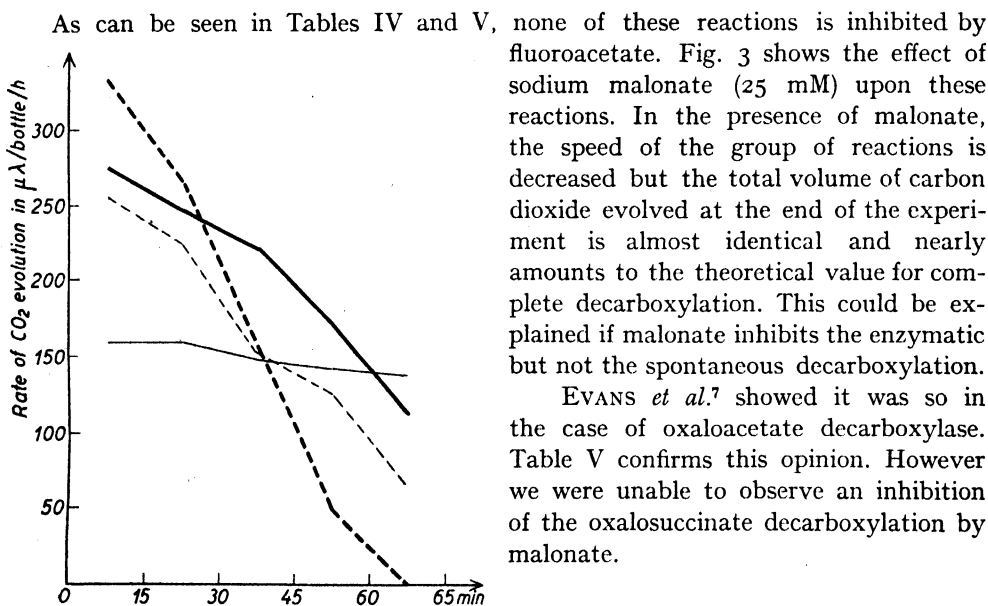


Fig. 3. Inhibition of the enzymatic decarboxylation of oxaloacetate and oxalosuccinate (provided as 3.3 mM fumarate and 3.3 mM citrate resp.) by 25 mM malonate

— Na fumarate  
— Na fumarate + malonate  
- - - Na citrate  
- - - Na citrate + malonate

#### ACCUMULATION OF CITRATE ACCOMPANYING THE INHIBITION BY FLUOROACETATE

The only part of the cycle which had not yet been investigated was the condensation step supposed to occur between a

"C<sub>2</sub> active fragment" derived from pyruvate by oxidative decarboxylation, and oxaloacetate, to give *cis*-aconitate.

TABLE VI

## FUMARATE AND CITRATE OXIDATION

Oxidation of sodium fumarate (6.6 mM) and sodium citrate (6.6 mM) by centrifuged kidney homogenate and inhibition by sodium fluoroacetate (3.3 mM); 2 h incubation at 38° C. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Exp.	Fumarate as substrate			Citrate as substrate		
	O <sub>2</sub> uptake (μl/bottle)		Inhibition	O <sub>2</sub> uptake (μl/bottle)		Inhibition
	Without NaFlAc	With NaFlAc		Without NaFlAc	With NaFlAc	
2696	407	267	34 %	503	333	32 %
2700	357	260	27 %	428	282	34 %

Since aconitase was shown to be insensitive to fluoroacetate, it should be possible to study the condensation reaction by means of citrate estimations. As can be seen in Table II, instead of a decreased citrate formation, a strong accumulation was observed, whereas citrate is well oxidised by a normal preparation (Table VI).

With various concentrations of fluoroacetate ranging from 0.66 mM to 33.3 mM (from 0.2 to 10.0 mg NaFlAc/bottle) the inhibition of O<sub>2</sub> uptake increases but the accumulation does not increase to the same extent (Table VII and Fig. 4).

Both inhibition (8–16 %) and accumulation of citrate can be observed with concentrations of fluoroacetate as minute as 0.05 mM (15 μg NaFlAc/bottle) that is to say the concentration required to kill a rat in 12–36 hours

(5 mg/kg). Fig. 5 shows that an accumulation of citrate increasing in parallel with the inhibition of the O<sub>2</sub> uptake, is observed between 0.05 and 0.5 mM.

Assuming that extrapolation from an acetone powder of pigeon liver to a homogenate of guinea pig kidney cortex is permitted, the problems seems to come to an apparent contradiction; apparently none of the reactions of the cycle is inhibited by fluoroacetate, whereas the cycle itself is interrupted somewhere below the citrate level.

It seems that the only hypothesis to explain this apparent deadlock is that, in the kidney preparation, fluoroacetate is not the inhibitor but that it is transformed into another substance which is inhibitory. This transformation does not occur in the liver acetone powder.

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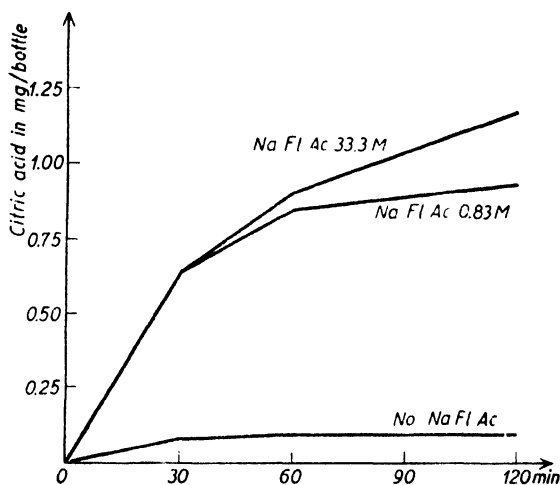


Fig. 4. Accumulation of citrate in the presence of fluoroacetate with sodium fumarate (6.6 mM) as substrate



A possibility which will be examined in the discussion is that fluoroacetate activated to a "fluoro  $C_2$  active fragment" enters the cycle and blocks it somewhere.

TABLE VII

COMPARISON BETWEEN THE INHIBITION BY DIFFERENT CONCENTRATIONS OF FLUOROACETATE OF THE OXYGEN UPTAKE AND THE CITRATE ACCUMULATION IN A CENTRIFUGED KIDNEY HOMOGENATE

Sodium fumarate (6.6 mM) as substrate; 2 hours' incubation at 38 %. All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate, 0.4 mM ATP.

Experiment	NaFlAc (mM)	Inhibition of the $O_2$ uptake	Citric acid (mg/bottle)
2672	0.00	—	0.27
	1.66	32 %	0.32
	16.6	64 %	0.47
2674	0.00	—	0.31
	1.66	37 %	1.30
	16.6	51 %	1.26
2677	0.00	—	0.09
	0.83	34 %	0.94
	33.3	50 %	1.18
2678	0.00	—	0.13
	0.83	—	0.92
	33.3	—	0.91

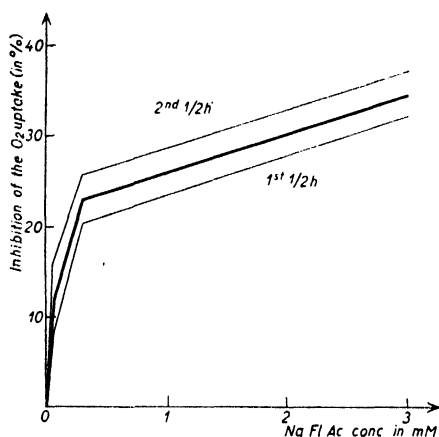
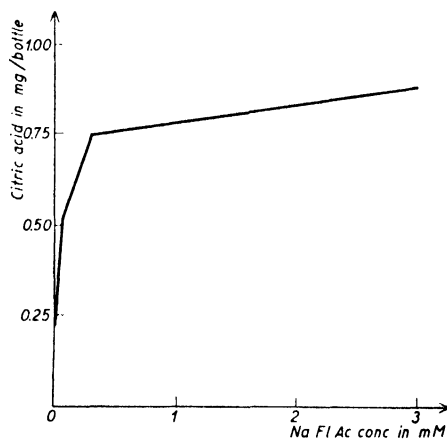


Fig. 5. a) Relation between inhibition and concentration of fluoroacetate

(In this experiment, the homogenate was centrifuged 45 min; boiling of the samples with  $H_2SO_4$  was performed before citric acid was estimated; 1 h incubation).



b) Relation between accumulation of citrate and concentration of fluoroacetate

The following experiments are consistent with this hypothesis.

The fact that very small amounts of fluoroacetate produce nearly the same accumulation of citrate as larger amounts, together with the fact (*vide infra*) that the factor bringing the acetate into the cycle is not very active in these preparations, suggest

that a very small amount of the added fluoroacetate only is activated and that we may deal with far less actual inhibitor than we think.

Whatever the interpretation of these results may be, the striking fact is that citrate accumulates and that acetate does not, a fact which cannot be explained by BARTLETT and BARRON's theory.

This accumulation of citrate is not inconsistent with the inhibition of the citrate synthesis observed by KALNITZKY AND BARRON<sup>26</sup> who used baker's yeast as enzyme preparation and acetate as substrate.

#### INHIBITION BY FLUOROACETATE WITH FUMARATE AND PYRUVATE AS SUBSTRATES

With sodium fumarate (6.6 mM) and sodium pyruvate (10 mM) as substrate, the O<sub>2</sub> uptake is 20 to 50% higher than with fumarate alone and there is an increased production of citrate even in the absence of fluoroacetate. The inhibition by fluoroacetate is lower and larger amounts of citrate accumulate than with fumarate alone. No significant amount of acetate accumulates under these conditions (Table VIII).

TABLE VIII  
CENTRIFUGED KIDNEY HOMOGENATE

Inhibition of the oxygen uptake and accumulation of citrate in the presence of sodium fluoroacetate (3.3 mM), with sodium fumarate (6.6 mM) and sodium pyruvate (10 mM) as substrates, 2 hours' incubation at 38° C. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Exp.	Substrate	O <sub>2</sub> uptake (μl/bottle)		Inhibition by NaFlAc	Acetic acid (mg/bottle)		Citric acid (mg/bottle)	
		Without NaFlAc	With NaFlAc		Without NaFlAc	With NaFlAc	Without NaFlAc	With NaFlAc
2685	F	462	275	40%	0.10 (± 0.05)	0.10 (± 0.05)	0.18	0.36
	F+P	545	461	15%	0.05 (± 0.05)	0.15 (± 0.05)	0.60	1.00
2699	F	436	246	43%	0.11 (± 0.03)	0.13 (± 0.03)	0.18**	0.75**
	F+P	561	406	27%	0.07 (± 0.03)	0.13 (± 0.03)	0.43**	2.02**
2700	F	375	260	27%	0.28 (± 0.03)	0.23 (± 0.03)	0.25	0.75
	F+P	532	389	27%	0.23 (± 0.03)	0.21 (± 0.03)	0.59	1.60
2672	F	272	184*	32%	—	—	0.26	0.32
	F+P	346	311*	10%	—	—	0.42	1.20

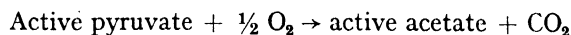
Figures between brackets mean the possible error.

\* NaFlAc 1.66 mM

\*\* Samples were boiled with H<sub>2</sub>SO<sub>4</sub> before estimation of citric acid.

F = fumarate; P = pyruvate.

This lifting of the inhibition by pyruvate may be only apparent. It is possible that "C<sub>2</sub> active fragment" formed from pyruvate competes with "fluoro C<sub>2</sub> active fragment" formed from fluoroacetate. A more likely alternative is that the increase of the O<sub>2</sub> uptake observed by addition of pyruvate is not due to a general increase of all the reactions of the cycle but to the increase of the step:



as the increased formation of citrate is evidence of it. Thus a larger proportion of the O<sub>2</sub> uptake is due to a reaction not inhibited by fluoroacetate.

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## INHIBITION BY FLUOROACETATE WITH FUMARATE AND ACETATE AS SUBSTRATES

With sodium fumarate (6.6 mM) and sodium acetate (10 mM) as substrates, the  $O_2$  uptake is slightly higher (4-11%) than with fumarate alone and there is more citrate present at the end of the incubation period (Table IX). This is interpreted as an indication that the factor responsible for bringing the acetate into the cycle is not very active in this kidney preparation. Usually the inhibition by fluoroacetate is very slightly lower as well as the accumulation of citrate, which is consistent with a competition at this stage between acetate and fluoroacetate, as envisaged in the theory of BARTLETT AND BARRON<sup>2</sup>. In one experiment (2696) in which the increase of citrate formation without fluoroacetate was slight, there was no change in the inhibition and no decrease in the accumulation of citrate when fluoroacetate was added. Not much stress can be laid on these experiments with acetate owing to the small changes, but they are consistent with BARRON's theory.

TABLE IX

CENTRIFUGED KIDNEY HOMOGENATE

Inhibition of the oxygen uptake and accumulation of citrate in the presence of sodium fluoroacetate (3.3 mM), with sodium fumarate (6.6 mM) and sodium acetate (10 mM) as substrates. 2 hours' incubation at 38° C. All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate, 0.4 mM ATP.

Experiment	Substrate	$O_2$ uptake ( $\mu$ l/bottle)		Inhibition by NaFlAc	Citric acid (mg/bottle)	
		Without NaFlAc	With NaFlAc		Without NaFlAc	With NaFlAc
2685	F	462	275	40 %	0.18	0.36
	F + A	510	309	40 %	0.32	0.26
2688	F	474	307	35 %	0.24	0.85
	F + A	528	374	29 %	0.33	0.70
2696	F	407	267	34 %	0.22	0.53
	F + A	378	254	35 %	0.29	0.56
2699	F	436	246	43 %	0.18	0.75
	F + A	454	296	34 %	0.23	0.53

F = fumarate; A = acetate

## DISCUSSION

*Inhibition of citrate oxidation.* The observations in this paper (excluding those in the appendix) have been made upon a finely ground homogenate, as distinct from organized tissue slice preparations; it was also freed from residual substrates. This renders interpretation somewhat easier. It has been shown on the basis of experiments by BARTLETT AND BARRON<sup>2</sup> by Webb (see DIXON AND NEEDHAM<sup>27</sup>), by ourselves (PETERS AND WAKELIN, unpublished) and this report, that none of the reactions of the tricarboxylic cycle yet studied separately can be considered responsible for the inhibition of the respiration of a homogenate of guinea pig kidney cortex, when fumarate is used as substrate. In order to explain the accumulation of citrate under these conditions, it has been proposed as a hypothesis that fluoroacetate, as well as acetate (BUCHANAN *et al.*<sup>20</sup>) can be activated and brought into the cycle. All the experiments published in

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this paper support this view though they do not prove it. It must be added that this conception is in perfect agreement with the fact known since fluoroacetate was prepared (SWARTS<sup>1</sup>) that the C-F bond is very stable and unreactive. The size of the fluorine atom is not much greater than the size of the hydrogen atom, and it can be expected that fluoroacetate might be metabolized to some extent as acetate, until a compound is reached, like fluoromalate for instance ( $\text{COOK-CH}_2\text{-CFOH-COOK}$ ) which cannot be dehydrogenated. Our hypothesis is in agreement with the fact shown by BARTLETT AND BARRON<sup>2</sup> that tissues or preparations unable to oxidize acetate are not inhibited by fluoroacetate, a fact which is confirmed in the case of the pigeon brain. It is also in accordance with the very interesting observations by SAUNDERS and collaborators (SAUNDERS<sup>28</sup>) who showed that among the substituted fluorofatty acids, only the even numbered were inhibitory, thus drawing the attention to the  $\text{CH}_2\text{F.CO}$  grouping, that is to say, to 'active fluoroacetate' rather than to acetate itself.

*Acetate.* In a pigeon brain homogenate, the active  $\text{C}_2$  fragment is stabilized as acetate in absence of added fumarate, (COXON, LIÉBECQ, AND PETERS<sup>29</sup>). The absence of accumulation of acetate in the kidney homogenate can be explained by the large excess of fumarate present, so that the  $\text{C}_2$  fragments formed from oxaloacetate via pyruvate are immediately used in synthesis. In one experiment with a homogenate, in which 30  $\mu\text{moles}$  of pyruvate and 20  $\mu\text{moles}$  fumarate were used as substrates, 15.65  $\mu\text{moles}$  of citrate were formed after 3 hours of incubation ( $\text{NaFlAc}$  0.33 mM). Since the inhibition by fluoroacetate is not immediately maximal, it may be assumed that a part of the citrate formed was oxidized; another part should be present as *cis*-aconitate and isocitrate which are not estimated by the method used. Thus the transformation of 20  $\mu\text{moles}$  fumarate to 15.65  $\mu\text{moles}$  citrate represents a nearly complete one. As far as we know these are the best conditions ever realized to prove the reality of the synthesis of citrate itself in animal tissues, which has been a disputed phase of the tricarboxylic cycle (BREUSCH<sup>30</sup>). This high citrate figure makes it clear that fluoroacetate or 'active fluoroacetate' does not interfere with the synthesis of citrate. Any possible competition between the ' $\text{C}_2$  active fragment' and the 'fluoro  $\text{C}_2$  active fragment' cannot be inhibitory.

*Pyruvate metabolism.* In regard to the relation of our observations to the degradation of pyruvate, the work upon the avitaminous brain supports the idea of the  $\text{C}_2$  intermediates. Our results are one more argument in favour of the formation of such a  $\text{C}_2$  active fragment from pyruvate followed by a condensation with oxaloacetate rather than the formation of a  $\text{C}_7$  compound pyruvate and oxaloacetate, which has had little experimental support.

*Relation to the biochemical lesion.* We have confirmed the observations in the literature that doses of 5 mg/kilo are toxic to rats (CHENOWETH AND GILMAN<sup>31</sup>); rats injected with this dose do not show signs for at least 30 min and usually die within 12-36 hours. The  $\text{LD}_{50}$  dose for the guinea pig is given as 0.35 mg/kg. In most cases we have used larger concentrations than this in our experiments *in vitro*, but it was found that a concentration of 0.05 mM (*i.e.*, 5 mg/kg) produced an inhibition of 16% in the second half hour of the experiment and a strong accumulation of citrate. These amounts are sufficiently close to the doses *in vivo* to suggest that the toxicity is really due to interference with the tricarboxylic cycle. It must be remembered that the proportion of fluoroacetate brought into the cycle according to our hypothesis is not known; from the fact that large amounts of fluoroacetate induced the same accumulation of citrate as

small amounts, it can be deduced that a limiting factor is involved in the activation of fluoroacetate. It is consistent with other evidence from arsenic poisoning that an attack upon the tricarboxylic cycle should induce a biochemical lesion, and this shows the outstanding importance of this cycle (KREBS AND JOHNSON<sup>23</sup>; KREBS<sup>24</sup>) in the intermediate metabolism. Since nervous signs are induced, the presence of the tricarboxylic cycle in brain can be inferred, for which direct evidence is given in another paper. It is interesting to note that BACQ<sup>32</sup> classed fluoroacetate among the "substances vésicantes" as it gave the LUNDGAARD effect in muscle.

*Specificity of malonate.* That malonate may not be a specific inhibitor of succinodihydrogenase is a view which has already been raised by WEIL-MALHERBE<sup>11</sup>. STARE AND BAUMANN<sup>33</sup> found that malonate 1 mM inhibited the oxidation of citrate in muscle tissue by 42% and that of succinate by only 16%. EVANS *et al.*<sup>7</sup> showed that the enzymatic decarboxylation of oxaloacetate was completely inhibited by 10 mM malonate. As has been shown in the experimental part of this paper, we were able to confirm this observation (using 25 mM malonate). Whether this is of importance when various substrates forming oxaloacetate are oxidized by the enzyme system is doubtful, because it would be expected that this inhibition would be lifted by addition of pyruvate. This was not always the case as can be seen in the experiment shown in Table X; the spontaneous decarboxylation seemed to be nearly sufficient in this system.

TABLE X

INHIBITION OF THE OXYGEN UPTAKE BY SODIUM MALONATE (25 mM) IN A CENTRIFUGED KIDNEY HOMOGENATE

Sodium fumarate (6.6 mM) and pyruvate (10 mM) as substrates. 2 hours' incubation at 38° C. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Experiment	Substrate	O <sub>2</sub> uptake (μl/bottle)		Inhibition by malonate
		Without Malonate	With Malonate	
2666	F	499	304	39 %
	F + P	594	370	38 %
2672	F	272	90	67 %
	F + P	346	153	56 %

F = fumarate; P = pyruvate

We failed to show the same inhibitory effect of malonate on the rather similar decarboxylation of oxalosuccinate. This is taken as one more evidence for the non-identity of the enzymes concerned. On the other hand, Fig. 3 shows that there is a slowing of the transformation of citrate to α-ketoglutarate when malonate was added. This confirms the point of STARE AND BAUMANN that malonate also acts somewhere between citrate and succinate and excludes the attack on the oxalosuccinate decarboxylation, which is mainly a spontaneous reaction under the conditions used, even more so than the decarboxylation of oxaloacetate.

(During the completion of this manuscript, we received the interesting paper by G. KALNITSKY, *Arch. Biochem.*, 17 (1948) 403, in which he has found using kidney tissue that fluoroacetate can induce an accumulation of citrate).

APPENDIX

*Experiments with kidney brei* (guinea pig). During the course of this research some experiments with a kidney brei have been made, which merit a brief note. The residual respiration of a kidney brei (unwashed) was inhibited about 50% by fluoroacetate; increases in acetate formed during this inhibition are slight and hardly significant, even not constant. In one experiment where very small amounts of fluoroacetate were used, as little as 0.05 mM sodium fluoroacetate produced a very marked accumulation of citrate (Table XI). The small changes in acetate accumulation and the poisoning are in agreement again with our view that the 'cycle' is attacked.

TABLE XI  
INHIBITION OF THE RESIDUAL RESPIRATION OF A KIDNEY BREI BY SODIUM FLUOROACETATE  
2 hours' Incubation at 38° C

Experiment	NaFlAc (mM)	O <sub>2</sub> uptake (μl/g tissue)	Inhibition by NaFlAc	Acetic acid (mg/g tissue)	Citric acid (mg/g tissue)
2613	0	2920	—	3.2	—
	15	1320	55 %	4.3*	—
2617	0	1840	—	0.2	—
	15	825	55 %	0.0	—
2618	0	2510	—	0.2	—
	15	1180	53 %	0.9	—
2697	0.00	2192	—	—	0.23
	0.05	2100	4 %	—	0.62
	0.15	1905	13 %	—	0.85
	0.50	1695	22 %	—	1.26
	3.00	1495	31 %	—	1.61
	15.0	1365	37 %	—	1.44

\* This accumulation of 1.1 mg acetic represents a variation of 0.05 mg acetic acid between the values actually measured, i.e., within the limits of error.

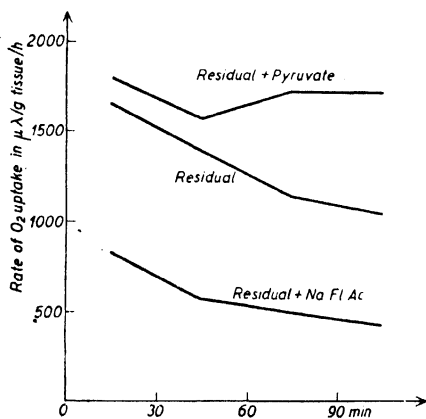
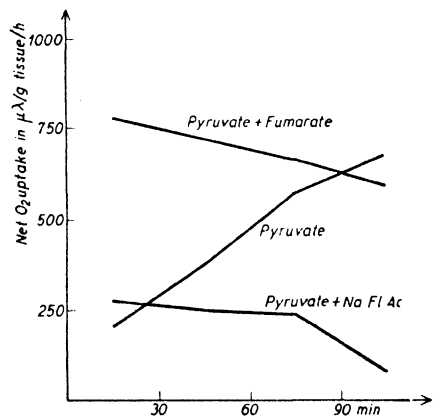


Fig. 6. a) Increase of the oxygen uptake due to the addition of sodium pyruvate (18 mM) to a brei of guinea pig kidney; inhibition of the residual respiration by sodium fluoroacetate (15 mM)



b) Net oxygen uptake due to the addition of sodium pyruvate (18 mM) and its inhibition by sodium fluoroacetate (15 mM), or due to the addition of sodium pyruvate (18 mM) and sodium fumarate (3.3 mM)

When pyruvate was added, a curious phenomenon was seen; the extra oxygen uptake due to the addition of pyruvate increased regularly. Fig. 6a shows that this was not a stabilization of the rate of oxygen uptake by the brei; the increase of the net  $O_2$  uptake due to pyruvate is inhibited by fluoroacetate (Fig. 6b). If fumarate was also present, the net oxygen uptake due to pyruvate plus fumarate was immediately maximal (Fig. 6b). These facts strongly suggest that the increase was due to the formation of fumarate from pyruvate, that the synthesis of this was inhibited by fluoroacetate. Unlike brain brei (BANGA *et al.*<sup>5</sup>), the kidney brei must be permeable to fumarate. These facts are consistent with the idea that the fumarate concerned is formed via the tricarboxylic cycle. It is more difficult to draw sharp conclusions from the brei as the residual respiration represents about 75% of the total respiration.

We are indebted to Dr B. C. SAUNDERS for a gift of sodium fluoroacetate; to the British Council for a Scholarship to one of us (C.L.), and to R. W. WAKELIN for skilful technical assistance.

#### SUMMARY

1. The hypothesis that fluoroacetate is a competitive inhibitor for acetate in tissue metabolism (BARTLETT AND BARRON) has been examined and found incapable of explaining some of the enzymatic effects of the poison.
2. Using a homogenate from guinea pig kidney, free from residual oxidizable substrates, and reinforced with  $Mg^{++}$  and adenine nucleotides, it was found that this readily oxidizes fumarate and citrate. In presence of fluoroacetate, citrate accumulates during the oxidation of fumarate, without an accompanying accumulation of acetate.
3. With the same homogenate, fumarate and pyruvate together give 20–50% increased oxygen uptake as compared with fumarate alone, and there is even formation of citrate in absence of poison. Fluoroacetate produces inhibition without accumulation of acetate, but this is less than with fumarate alone.
4. Fluoroacetate has no action upon the pyruvate dehydrogenase component of the brain pyruvate oxidase system. It also has no effect upon the activity of the enzymes aconitase, oxalosuccinate decarboxylase, fumarase and oxaloacetate decarboxylase, as tested by a pigeon liver preparation.
5. The fact that no single enzymatic reaction has been found to be inhibited by fluoroacetate and yet that the reactions of the tricarboxylic cycle are stopped with accumulation of citrate requires a hypothesis in addition to that proposed by BARRON AND BARTLETT.
6. It is suggested that, in the kidney preparation, fluoroacetate is not the inhibitor but that it is transformed into another substance which is inhibitory.
7. The accumulation of citrate can be observed with concentrations of 0.05 mM (15  $\mu g$  Nafluoroacetate per bottle) which approximates to the amounts causing toxic effects *in vivo*.
8. It was confirmed that malonate inhibits the enzymatic decarboxylation of oxaloacetate.
9. Some experiments upon kidney brei are described.

#### RÉSUMÉ

1. Nous avons examiné l'hypothèse proposée par BARTLETT ET BARRON, selon laquelle le fluoroacétate inhibe le métabolisme tissulaire par compétition avec l'acétate, et avons constaté qu'elle était incapable d'expliquer certains effets du poison sur les réactions enzymatiques du cycle des acides tricarboxyliques.
2. Un "homogénat" de rein de Cobaye, dépourvu de substrats oxydables, oxyde parfaitement le fumarate et le citrate, pour autant qu'on y ajoute des nucléotides d'adénine et des ions  $Mg^{++}$ . En présence de fluoroacétate, du citrate s'accumule au cours de l'oxydation du fumarate, sans accumulation simultanée d'acétate.
3. Au contact de fumarate et pyruvate au lieu de fumarate seul, la même préparation enzymatique consomme 20 à 50% d'oxygène supplémentaire et la formation de citrate est nettement accrue, même en l'absence de tout inhibiteur. Le fluoroacétate produit une inhibition de la consommation d'oxygène (sans accumulation d'acétate), moindre cependant qu'au contact de fumarate seul.

4. Le fluoroacétate n'a aucune action sur la déshydrogénase pyruvique, une des pièces du système oxydase pyruvique du cerveau. Il n'a pas plus d'action sur l'aconitase, la décarboxylase de l'oxalosuccinate, la fumarase et la décarboxylase de l'oxaloacétate, enzymes étudiées à l'aide d'une poudre de foie de Pigeon.

5. Le fait qu'aucune réaction enzymatique isolée ne s'est montrée sensible à l'action du fluoroacétate, mais que d'autre part le cycle des acides tricarboxyliques est bloqué avec accumulation de citrate, nécessite une hypothèse supplémentaire à celle de BARTLETT ET BARRON.

6. Nous suggérons que, dans les préparations de rein utilisées, le fluoroacétate lui-même n'est pas l'inhibiteur, mais qu'il est transformé en une autre substance, responsable de l'inhibition.

7. On peut observer l'accumulation de citrate avec des concentrations de fluoroacétate de 0.05 mM (15  $\mu$ g de fluoroacétate sodique par auge), c'est-à-dire une concentration provoquant l'intoxication *in vivo*.

8. Nous confirmons l'inhibition de la décarboxylation enzymatique de l'oxaloacétate par le malonate sodique.

9. Nous décrivons quelques expériences faites avec une pulpe de cortex rénal.

### ZUSAMMENFASSUNG

1. Die Hypothese, dass Fluoracetat ein Hemmstoff für Acetat im Gewebestoffwechsel sei (BARTLETT UND BARRON), wurde geprüft. Dabei wurde festgestellt, dass die Hypothese nicht imstande ist, einige enzymatische Wirkungen des Giftes zu erklären.

2. Bei Benutzung eines "Homogenats" von Meerschweincheniere, das frei von oxydierbaren Restsubstraten war und mit  $Mg^{++}$  and Adeninnukleotiden verstärkt war, wurden Fumarat und Citrat schnell oxydiert. Bei Anwesenheit von Fluoracetat wird während der Fumaratoxydation Citrat angehäuft, und zwar ohne dass diese Anhäufung von einer Anhäufung von Acetat begleitet wäre.

3. Bei demselben Enzympräparat ergeben Fumarat und Pyruvat zusammen eine Erhöhung der Sauerstoffaufnahme von 20–50 % im Vergleich mit Fumarat allein, und bei Abwesenheit des Giftes tritt sogar Citratbildung auf. Fluoracetat verursacht eine Hemmung ohne Anhäufung von Acetat, diese Hemmung ist jedoch geringer als bei Fumarat allein.

4. Fluoracetat hat keinen Effekt auf die Pyruvatdehydrogenasekomponente des Pyruvatoxydationssystems des Gehirns. Es hat auch keine Wirkung auf die Aktivität der Enzyme Aconitase, Oxalsuccinatdecarboxylase, Fumarase und Oxalacetatdecarboxylase, wie mit einem Taubenleberpräparat geprüft wurde.

5. Die Tatsache, dass, wie festgestellt, keine einzelne enzymatische Reaktion durch Fluoracetat gehemmt wurde, und dass doch die Reaktionen des Tricarbonsäurecyklus unter Anhäufung von Citrat gehemmt wurden, erfordert noch eine Hypothese neben der von BARTLETT UND BARRON.

6. Es wird angenommen, dass bei dem Nierenpräparat nicht Fluoracetat der Hemmstoff sei, sondern dass dieses in eine andere Substanz umgesetzt wird, die eine hemmende Wirkung hat.

7. Die Citratanhäufung kann bei Konzentrationen von 0.05 mM (15  $\mu$ g NaFluoracetat pro Gefäß) wahrgenommen werden, was in der Nähe der Mengen, die die toxischen Wirkungen *in vivo* ausüben, liegt.

8. Es wurde bestätigt, dass Malonat die enzymatische Decarboxylierung von Oxalacetat hemmt.

9. Einige Versuche mit Nierenbrei werden beschrieben.

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#### NOTE ADDED AT CORRECTION

In regard to Fig. 2, while our paper was in the press, MEHLER, KORNBERG, GRISOLIA, AND OCHOA, *J. Biol. Chem.*, 174 (1949) 961, showed that some reactions with the pigeon liver preparation take a different course and include TPN; this does not seem to upset our arguments. There also appeared a further report by KALNITZKY AND BARRON, *Arch. Biochem.*, 19 (1948) 75; working with undialysed rabbit kidney preparation, they have observed an increased synthesis of citrate in presence of fluoroacetate; in our work, the reduction of the residual respiration makes interpretation less difficult.

## THE EFFECT OF ADRENALINE ON THE UTILIZATION OF GLUCOSE

by

J. A. COHEN

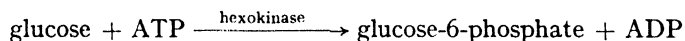
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### INTRODUCTION

During the last 15 years there has been some controversy as to the effect of adrenaline upon glucose utilization by the tissues. CORI (1928 a b c) is of the opinion that the tissues (of which muscle is quantitatively the most important) show lowered utilization after adrenaline injection. After glucose absorption rats can use 250 mg glucose/h for several hours; after adrenaline injection the glucose coming from the liver was calculated to be only about 50 mg/h; yet this was enough to cause prolonged hyperglycaemia. Other authors arrived at the same conclusion (LUNDGAARD, 1938; CONN *et al.*, 1940; DILL *et al.*, 1939; COURTICE, 1939; WIECHMANN, 1927).

This view of the effect of adrenaline has been contested, *e.g.*, by SOSKIN (1927) and his colleagues, who used measurements of blood flow and converted arteriovenous bloodsugar differences into amounts of sugar retained by the muscles per unit of time. Their results were confirmed by the work of other authors (*e.g.*, HIMSWORTH AND SCOTT, 1927; JONKERS, 1945). They found no evidence for an effect of adrenaline on glucose utilization.

Since experimental work on this subject has all been done *in vivo* and the relevant data were usually obtained by calculation involving approximations and assumptions, it seemed of interest to try to obtain some more direct information from experiments *in vitro*. Rat diaphragm was used for these experiments on the assumption that results obtained on this object would be representative for muscle in general. The glucose utilization investigated and referred to in this paper was regarded as the uptake of sugar by the tissues, thus being governed by the intrinsic activities of the enzyme systems responsible for this uptake at a constant supply of substrate. More explicitly this is taken to mean the rate of the reaction:



assuming that this reaction determines the rate of glucose uptake. This reaction was followed by determining the rate of anaerobic glycolysis of diaphragm on the assumption that the rate of glycolysis is limited by the hexokinase reaction.

### EXPERIMENTAL METHODS

Groups of 4 animals were used of which 2 were injected and 2 served as controls. They were all starved for a period of 19–24 hours before the experiment. They were killed by decapitation 40–70 minutes after the injection of adrenaline into the experimental animals of the group. Littermates

*References p. 241.*

(weighing 100–120 g) were used for the great majority of the experiments. It proved necessary to standardize the conditions rigorously from the moment the animals were killed. It was so arranged that the first manometer reading was performed exactly 35 min after the first animal of the group of 4 had been killed. Two of every group of 4 animals provided tissue for the 2 control manometers (with and without glucose); the remaining 2, which had been injected with adrenaline, furnished tissue for the 2 experimental manometers (with and without glucose). Thus every manometer received approximately equal portions of diaphragm from each of 2 identically treated animals. The 4 diaphragms were taken out and quickly washed in KREBS-HENSELEIT salt solution. The membranaceous part was dissected out and discarded. They were then divided, distributed and placed in 4 manometers. Two of these manometers contained KREBS-HENSELEIT-RINGER bicarbonate solution with 0.2% glucose. The 2 others contained the same solution without glucose. Thus the final distribution over the manometers was, that the diaphragms of the adrenaline injected and the control rats were tested both in the presence and in the absence of glucose. BARCROFT manometers were used. The gaseous medium was  $N_2$  containing 5%  $CO_2$  to maintain a pH with the bicarbonate in the fluid medium of 7.4. The bath temperature was 38° C.

For the purpose of determination of phosphate hydrolysis curves 2 rats weighing approx. 100–120 g were killed and the diaphragms taken out as usual. These were weighed on a torsion balance and transferred at once to a cooled mortar and immersed in 5 ml trichloroacetic acid (T.C.A.) (5%). After crushing in the mortar this extract was filtered through a Gooch funnel into a cooled flask and the residue washed with 5 ml T.C.A. After neutralization to phenolphthalein with sat. soda the volume was made up to 15 ml (stock solution). 1.5 ml of the stock solution was used for the determination of inorganic plus creatine phosphate. This was estimated by diluting this volume of the stock solution and adding the calculated amount of ammonium molybdate- $H_2SO_4$ -reagent (1.5 ml) of the FISKE AND SUBARROW reaction. After standing for 20 min 0.6 ml of the FISKE SUBARROW reducing reagent was added, the volume made up to 15 ml and the phosphate estimated in the colorimeter. To 3 other samples of 1.5 ml of the stock solution were added 0.75 ml of 3N HCl. These samples were hydrolysed in a boiling water bath for periods of 7, 15 and 180 minutes. Total phosphate was determined in a sample of 1.5 ml using  $H_2SO_4$  for digestion followed by addition of 1 drop of nitric acid. All phosphates were determined using the FISKE AND SUBARROW method.

Adrenaline was injected subcutaneously 40–70 min before the experiment in a dose of 1 mg/kg rat and in a volume of approximately 0.5 ml. It was made up by dissolving adrenaline (base) in water acidified with HCl.

#### EXPERIMENTAL RESULTS

##### *Glycolysis of diaphragm of rats after treatment with adrenaline*

Anaerobic glycolysis was determined in diaphragms of rats injected with adrenaline and of untreated control rats. Fig. 1 gives a clear picture of the type of result that is usually obtained under the experimental conditions which have just been described in the technical part. Studying this figure, it will be seen at once that there is an inhibition of glycolysis in the diaphragm of the animals which had been treated with adrenaline. At first sight this inhibition seems to apply to the glycolysis in the absence as well as in the presence of glucose; closer examination of a great number of curves however reveals that most of the differences between treated and untreated diaphragms in these blanks (in the absence of glucose) disappear after an initial period of incubation of app. 10–20 min. In other words the small overall difference found between blanks from treated and untreated animals is usually generated in the first period only; after that period, when glycolysis becomes very low in either case, a significant difference no longer exists between the rates of glycolysis in blanks from diaphragms of treated and untreated animals. Faced with the existence of an initial glycolysis in the blanks which practically disappeared after 20 min and which differed slightly in treated animals and controls it was necessary to allow for the possibility that this difference would, to a certain extent, account for the differences found between treated animals and controls in the presence of glucose. It is the object of these experiments to interpret glycolysis of added glucose in terms of glucose uptake. If therefore the difference in glycolysis

rate between controls and treated animals in the presence of glucose were no greater than the difference observed in the absence of glucose, this difference in the presence of glucose would have no significance. Since the difference in the rate of glycolysis in blanks from treated animals and controls disappears after 20 min when glycolysis becomes minimal in both cases, it was decided to estimate the glycolysis in all experiments (involving diaphragms of treated animals and controls with and without glucose) during the period of 30 min elapsing between 20 min and 50 min from the commencement of incubation. The rate of glycolysis during that period served as a basis for the calculation of the conventional  $Q_{CO_2}^{N_2}$ . The figures thus obtained ought to reflect the true utilization of added glucose. The blanks of diaphragm from treated and untreated animals were the same under these conditions.

Tables I and II show the results of a series of experiments in which  $Q_{CO_2}^{N_2}$  has been thus computed. Table I shows that a significant difference between the blanks of both groups of animals no longer exists, whereas

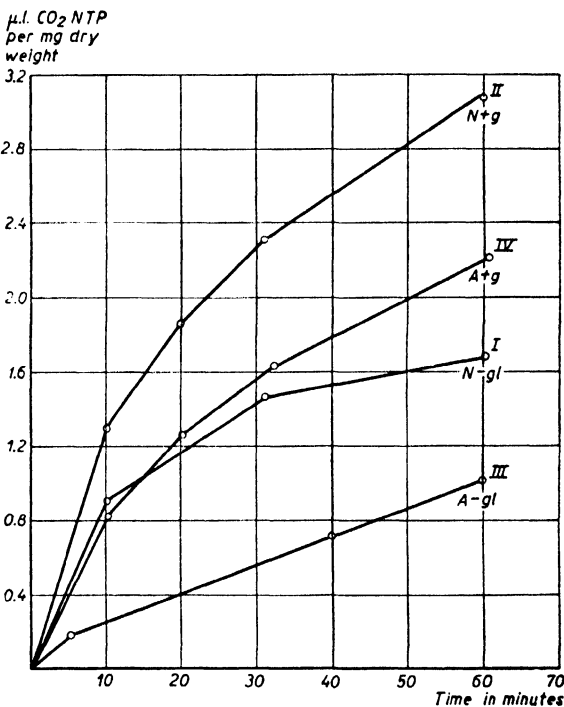


Fig. 1. Glycolysis of rat diaphragm under the influence of injected adrenaline. Curve I: normal blank; Curve II: normal + glucose; Curve III: adrenaline injected blank; Curve IV: adrenaline injected + glucose

TABLE I  
ANAEROBIC GLYCOLYSIS OF RAT DIAPHRAGM AFTER INJECTION OF ADRENALINE

Exp. No.	Control rats			Rats injected with adrenaline 1 mg/kg subcutaneously		
	Blanks	In presence of 2 mg glucose per ml	Difference due to glucose utilization	Blanks	In presence of 2 mg glucose per ml	Difference due to glucose utilization
1	1.06	3.00	1.94	1.06	2.40	1.34
2	0.64	2.32	1.68	0.60	1.92	1.32
3	1.00	3.36	2.36	0.76	1.58	0.82
4	1.34	2.38	1.04	0.80	1.68	0.88
5	0.68	1.52	0.84	0.94	1.60	0.66
6	0.78	2.40	1.62	0.70	1.74	1.04
7	0.92	2.08	1.16	0.92	1.34	0.42
8	1.26	3.00	1.74	0.96	1.60	0.64
9	0.76	1.48	0.72	0.56	1.84	1.28
10	0.84	1.90	1.06	0.10	1.46	1.36
Mean:	0.92	2.34	1.41	0.74	1.71	0.97

The inhibition of glucose utilization in the treated animals is 31 %. The figures represent  $Q_{CO_2}^{N_2}$ .

References p. 241.

there is still a clear-cut difference between the glycolysis of diaphragm from animals treated with adrenaline compared with the controls. The inhibition achieved by the adrenaline is approx. 30-36%. The spread in the results is considerable but highly significant differences were obtained when the results within one experiment (using littermates at the same day under the same conditions) were compared. Every figure represents the mean glycolysis of 2 diaphragms.

Table II summarizes a series of experiments of the same type as those in Table I, but here no blanks were determined because it had been found that no significant difference exists between blanks of normal and treated diaphragm, so that they could be dispensed with for the calculation of the absolute difference in glucose utilization between diaphragms of treated and untreated animals. For the exact calculation of the percentage inhibition of glucose utilization the blanks should be known. In the case of Table II the blanks from Table I have been applied. If this is done the percentage inhibition calculated amounts to 36%. When the inhibition is calculated in relation to the overall glycolysis (including blank glycolysis) it is found to be 31%.

TABLE II  
ANAEROBIC GLYCOLYSIS OF RAT DIAPHRAGM AFTER INJECTION  
OF ADRENALINE

Exp. No.	Control rats	Rats injected with adrenaline 1 mg/kg
11	3.6	2.2
12	2.04	1.96
13	2.10	2.2
14	2.72	1.44
15	1.90	1.4
16	1.84	1.3
17	3.26	1.84
18	2.76	1.74
Mean:	2.52	1.76

The difference between controls and treated animals =  $0.76 = 31\%$  of the overall glycolysis in the controls.

The figures represent  $Q_{CO_2}^N$ .

Inhibition of glucose utilization (using the blank values of Table I) = 36% of the glucose utilization in the controls.

In a few experiments the lactic acid production was followed chemically and compared with the manometric results. There was good agreement. The inhibition of glycolysis after adrenaline injection was also clearly reflected in the results of the chemical estimations. Lactic acid was estimated by the method of LE PAGE (UMBREIT *et al.*, 1946).

#### *Phosphate fractions in diaphragm*

Earlier in this paper it was shown that a certain amount of acid production occurs in the absence of glucose particularly in the first 20 min. This phenomenon was assumed to be due to lactic acid production from some intrinsic substrate presumably either glycogen or a carbohydrate phosphate. It seemed interesting for the interpretation of the glycolysis curves obtained to try and get an idea about the presence of intrinsic substrates by direct estimation of phosphate fractions and glycogen. Comparison of figures from phosphorylated intermediates in diaphragms from normal and adrenaline

injected animals might reveal such accumulation. Phosphorylated intermediates were estimated by carrying out phosphate hydrolysis curves, inorganic (incl. creatine phosphate) and total phosphate estimations. Results are given in Table IIIA, B and C.

TABLE IIIA  
PHOSPHATE FRACTIONS OF NORMAL AND ADRENALINE INJECTED ANIMALS

Normal animals	Inorganic + creatine phosphate P	7 min P	15 min P	3 h P	Total P
1	46.1	68.6	73.6	73.5	102.2
2	48.8	67.5	72.8	—	92.5
3	32.2	46	48.2	64	69
Adrenaline treated animals	Inorganic + creatine phosphate P	7 min P	15 min P	3 h P	Total P
1	51.4	69	70	80	103
2	56.6	71.3	72.1	79	91.7
3	42.3	52.3	55.3	59.7	81.6
4	51.8	73.3	72	86	98

The figures express phosphate in mg/100 g diaphragm.

7' P, 15' P etc. figures represent the total of inorganic P estimable after the corresponding periods of hydrolysis at 100° C.

TABLE IIIB  
PHOSPHATE FRACTIONS IN DIAPHRAGM OF NORMAL AND ADRENALINE INJECTED ANIMALS

Normal animals	Inorganic + creatine phosphate P	7 min P	15 min P	3 h P	Total P
1	45	67	72	72	100
2	53	73	79	—	100
3	47	67	70	93	100
Adrenaline treated animals	Inorganic + creatine phosphate P	7 min P	15 min P	3 h P	Total P
1	50	67	67	77	100
2	62	77	77	86	100
3	51	63	67	73	100
4	53	74	72	87	100

The figures express percentages of total phosphate content in diaphragm.

From Table IIIA it follows that no significant difference exists between treated animals and controls as regards the absolute amounts of the various phosphate fractions. From Table IIIB it may be concluded that the distribution of the phosphate fractions has not changed either after injection of adrenaline\*.

\* When from the figures of Table IIIA differences between the various fractions are calculated (Table IIIC) there seems to be a difference between the P 15-P 7 fractions from normal and adrenaline treated animals, which cannot be interpreted.

TABLE IIIC

TABLE OF DIFFERENCES OF VARIOUS PHOSPHATE FRACTIONS (CALCULATED FROM TABLE IIIA)

Normal animals	P 7-P 0	P 15-P 7	P 180-P 15	Pr-P 180
1	22.5	5.0	0.1	28.7
2	18.7	5.3	—	—
3	13.8	2.2	15.8	5.0
Adrenaline treated animals	P 7-P 0	P 15-P 7	P 180-P 15	Pr-P 180
1	17.6	1.0	1.0	23.0
2	14.7	0.8	6.9	12.7
3	10.0	3.0	4.4	21.9
4	21.5	—1.3	14.0	12.0

The figures represent mg P/100 g diaphragm.

### Glycogen estimations

Glycogen was estimated in the diaphragm of normal and adrenaline treated animals.

The method used was the PFLÜGER method as modified by SOMOGYI (GOOD, CRAMER AND SOMOGYI, 1933). Reduction after final hydrolysis was measured by the ceric sulphate method (MILLER AND VAN SLYKE, 1936). A group of 8 animals starved for a period of approx. 20 hours was used: 4 were injected with adrenaline, 4 served as controls. The animals were killed and the diaphragms taken out. The pooled diaphragms of every group of 4 were mixed and divided into 4 portions; every portion contained approximately equal amounts of each contributing diaphragm. The 4 portions of each pool, namely pool A (normal diaphragm) and pool B (diaphragm from treated animals) were dealt with as follows: the first 2 portions of each pool were placed directly into 30% KOH for glycogen estimations. The 2 remaining portions of each pool were incubated anaerobically as in the glycolysis experiments, one portion serving as a blank, the other in presence of glucose; after the incubation glycogen was estimated.

The results of a typical experiment are summarized in Table IV.

TABLE IV

GLYCOGEN BREAKDOWN IN DIAPHRAGM OF ANIMALS INJECTED WITH ADRENALINE AND CONTROLS DURING ANAEROBIC GLYCOLYSIS

Normal diaphragm			Diaphragm from adrenaline injected animals		
Initial	After incubation		Initial	After incubation	
	with glucose	without glucose		with glucose	without glucose
0.20 } 0.22 }	0.15	0.12	0.19 } 0.19 }	0.17	0.18

The figures represent % glycogen.

The table shows:

1. That there is no essential difference in initial glycogen level between the diaphragms of treated animals and controls, and that a low glycogen level cannot therefore be connected with the lowered glycolysis in diaphragms from adrenaline treated animals.
2. That the diaphragms of normal animals used 0.9 g of glycogen/100 g of muscle in the absence of glucose but only 0.6 g in the presence of glucose.

References p. 241.

3. The diaphragm of adrenaline treated animals used practically no glycogen when incubated either with or without glucose.

*Experiments with other substrates and tissues*

In a few experiments carried out on diaphragm in order to assess in what stage the anaerobic glycolysis was affected, hexose-6-phosphate and hexose diphosphate were used as substrates. No utilization took place of hexose-6-phosphate, whereas hexose diphosphate was utilized. The failure of diaphragm to utilize hexose monophosphate confirmed similar results of DIXON AND NEEDHAM (Personal Communication) on rat skin glycolysis. The method of determination of the anaerobic glycolysis in presence of these phosphates was the same as that employed with glucose. The substrate concentration was M/100.

In two experiments no difference was found in the utilization of hexose diphosphate in diaphragm of adrenaline injected animals and controls, indicating an effect of adrenaline prior to the action of zymohexase. The number of experiments is far too small to draw definite conclusions.

In 3 experiments the glycolysis of rat heart muscle slices was investigated. On this tissue the blank glycolysis in the absence of glucose is practically nil, whereas in the presence of glucose there is a considerable glycolysis. It was highly interesting to find that in all three experiments the glycolysis of the heart of the rats injected with adrenaline was considerably *higher* than of the controls.

Normal controls	Adrenaline treated animals
$Q_{CO_2}$	$Q_{CO_2}$
10.4	16.7
8.8	10.0
11.3	14.1

The inhibition of glucose utilization seen in other muscle (diaphragm) after adrenaline seems in the heart to be prevented if not reversed. This state of affairs is highly favourable to meet the particular stress which is exerted on the heart after adrenaline administration.

*Effect of the addition of adrenaline on the anaerobic glycolysis of diaphragm in vitro*

Adrenaline in a dose of 50–100  $\mu$ g was added from a KEILIN tube to normal diaphragm glycolysing in BARCROFT manometers under the usual conditions. Fig. 2 shows that no effect whatsoever is observed on the rate of glycolysis by the addition of adrenaline (at the arrow). It is therefore impossible to show any direct effect of adrenaline on the utilization of glucose by rat diaphragm under the conditions of the experiment.

# DISCUSSION

The most significant conclusion that can be drawn from the experiments described is that the peripheral utilization of glucose is decreased after injection of adrenaline (1 mg/kg) by 30% approximately as compared with the controls. This decrease is tentatively taken to be the results of an impaired hexokinase reaction which may be due to inhibition of the enzyme itself or to insufficiency of other factors determining the rate



of the reaction (e.g., ATP-level). The possibility of a decrease of lactic acid production after adrenaline treatment due to inhibition beyond the hexokinase reaction has not yet been excluded in these experiments. It will be expounded in a following paper on muscle extracts.

The fact that adrenaline added *in vitro* to the diaphragm had no inhibitory effect suggested an indirect action, the possible nature of which is dealt with in a separate paper.

The phosphate and glycogen estimations show that the differences found in the glycolysis of diaphragm of adrenaline treated animals and controls are not due to different quantities of intrinsic metabolite present before incubation.

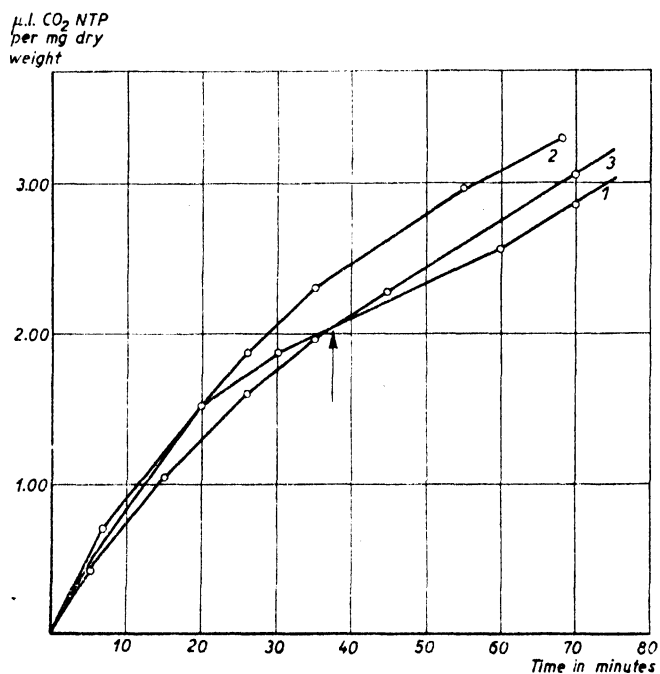


Fig. 2. Glycolysis of rat diaphragm under influence of adrenaline *in vitro*. In curve No. 1 adrenaline was added at the arrow in a dose of 50  $\mu\text{g}/\text{ml}$ . In curve No. 2 adrenaline was added at zero time in a dose of 100  $\mu\text{g}/\text{ml}$ . In curve No. 3 no adrenaline was added.

The absence in the phosphate experiments of increased accumulation of hexose-6-phosphate after adrenaline as found by CORI (1931) in the gastrocnemius, is probably due to the blurring out of this effect by the massive breakdown of glycogen into hexose phosphate in treated animals as well as in controls during the killing when no anaesthesia is used, demonstrated by the same author.

Neither can differences in the utilization of intrinsic substrates be held responsible for the marked differences observed in the glycolysis of diaphragms from adrenaline treated animals compared with the controls in the presence of glucose. This conclusion emanates from the experiments indicating that in the absence of added substrate, the presence of these intrinsic substances is not able to account for anything but a slight insignificant difference between the glycolysis of diaphragms of both groups of animals. The conclusion is based on the assumption that the breakdown of intrinsic substrate

and its inhibition after adrenaline is not larger in the presence than it is in the absence of added glucose. This assumption is certainly valid as far as glycogen is concerned since the difference in the rate of breakdown of this substance between diaphragms of treated and untreated animals has been shown to be even smaller in presence than it is in the absence of glucose.

On the strength of these arguments it appears that the inhibition of glycolysis after adrenaline in the experiments described in this paper is not dependent on differences in pre-existing quantities of intrinsic substrate but affects only the metabolism of added glucose.

The results of the glycogen estimations suggest that the slight difference found in glycolysis experiments between the blanks of treated animals and controls in the first 20 minutes of incubation may be due to a more intense glycogen breakdown in the normal animals as compared to those treated with adrenaline\*. The presence of glucose seems to inhibit the breakdown of glycogen during anaerobic incubation in diaphragm from the controls. It seems surprising that glycogen should be broken down better in the diaphragm from control animals than from the treated ones. This effect, however, is not constant and may easily be spurious because the number of experiments so far done is too small to allow a conclusion involving the comparison of phenomena in *different* mixtures of diaphragm (that is composed of tissue slices from *two* groups of animals). The inhibition of glycogen breakdown by glucose in the controls was established by comparison of tissue mixtures of identical composition (that is mixtures composed of tissue slices from one and the same group of animals) on three occasions and is therefore considered to be real. It has been demonstrated previously by other authors (SOSKIN, 1939).

It may be that some tissues containing hexokinase may not react to adrenaline according to the usual pattern. The few experiments on the glycolysis of heart slices recorded show that in the heart the hexokinase is not inhibited after adrenaline injection. Obviously the strain on the heart in its hyperactive state after adrenaline is enormous and it may well be that here a mechanism is operating preventing the usual inhibition of hexokinase in order to secure a steady flow of glucose into the organ. Such a discrepancy between the responses to adrenaline by heart muscle compared to other muscle is by no means unique. It will be remembered that, whereas adrenaline usually has a constricting effect on the arterial system in the body, it has a tendency to dilate the coronary arteries of the heart. Constriction would have led to poor feeding of the hyperactive organ which would have created a highly unfavourable condition. A mechanism is introduced which secures a steady flow of the necessary requirements to the active organ.

The physiological significance of the inhibition of the hexokinase reaction under conditions of emergency accompanied by release of adrenaline, is still obscure. A possible course of events is the following: When the body is called upon for quick emergency action, adrenaline is produced and as a result of this the breakdown of glycogen in the muscle is increased. This mechanism for providing energy produces more ATP per glucose residue than the hexokinase reaction. The latter reaction would interfere with the former in as far as it uses ATP for its initial phosphorylation. This interference is prevented by inhibition of hexokinase by adrenaline (indirectly) and glycogen breakdown can proceed undisturbed.

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\* An accompanying manometric experiment showed a good agreement between glycogen disappearance and lactic acid production.

An alternative possibility would be that after injection of adrenaline ATP is used for reactions closely related to mechanical muscle contraction. Less ATP would then be available for the hexokinase reaction. The so called *increased* glycogen breakdown may in this conception very well consist of a *decrease* in aerobic glycogen-synthesis, also caused by the lack of available ATP.

Whatever may be the case, obviously the glycogen reserves would quickly get exhausted particularly in those organs which like the heart are very active after release of adrenaline. Possibly in such organs no inhibition of the hexokinase reaction takes place. Moreover the blood sugar concentration rises after a short while so that an *increased* glucose utilization in such organs can take place after an initial period under the influence of the now prevailing hyperglycaemia, whereas the initially *decreased* glucose utilization as a result of the inhibition of the hexokinase reaction in other muscles etc. will now be compensated for by the high level of blood sugar making replenishments of stores possible even in the presence of a still impaired hexokinase activity or ATP level. These replenishing processes occur after an initial period during which the hyperglycaemia develops.

#### SUMMARY

1. This work was undertaken with the object of investigating whether the rise in blood sugar after adrenaline may at least be partly due to inhibition of glucose uptake by the tissue: more explicitly whether the hexokinase reaction determining the rate of glucose uptake by the tissues from the blood was impaired directly or indirectly after injection of adrenaline.

2. There is a marked inhibition of the anaerobic glycolysis of diaphragms from rats which have been injected with adrenaline (1 mg/kg subcutaneously) 40-70 minutes previously. This inhibition is approximately 30-36%. The manometric result could be confirmed by chemical lactic acid estimations. The inhibition does not occur in the blank estimations in the absence of glucose.

3. When various phosphate fractions like inorganic P (incl. creatine P), 7 min P, 15 min P, 180 min P and Total P were determined in diaphragms from adrenaline injected rats and compared with normal controls, no significant differences could be demonstrated.

4. There is no essential difference in initial glycogen level between the diaphragms of treated animals and controls. The presence of glucose during anaerobic incubation seems to inhibit glycolysis in diaphragm from normal animals. It appears that quantitative differences in intrinsic substrate (glycogen, phosphorylated carbohydrate) cannot be responsible for the marked inhibition observed in the glycolysis of diaphragms from adrenaline treated animals compared with the controls in the presence of glucose. This inhibition only affects the metabolism of added glucose.

5. When hexose diphosphate is used as a substrate no inhibition of the anaerobic glycolysis of diaphragm from adrenaline treated animals is observed suggesting the inhibiting action towards glucose to occur prior to the zymohexase reaction in the glycolysis chain.

6. A direct inhibition of glucose utilization of diaphragm under influence of adrenaline added *in vitro*, cannot be demonstrated.

#### RÉSUMÉ

1. Le but de ce travail était de rechercher si l'augmentation du sucre sanguin par l'adrénaline est due, partiellement au moins, à l'inhibition de l'absorption du glucose par les tissus; ou, d'une façon plus précise, si l'action de l'hexokinase qui détermine la vitesse de l'absorption du glucose à partir du sang par les tissus, est plus ou moins directement bloquée après injection d'adrénaline.

2. Il se manifeste une inhibition marquée de la glycolyse anaérobie du diaphragme de rats ayant reçu de l'adrénaline par injection sous-cutanée (1 mg/kg) 40-70 minutes auparavant. Cette inhibition est de l'ordre de 30-36%. Les résultats obtenus par voie manométrique ont pu être confirmés par des dosages d'acide lactique. Cette inhibition ne se manifeste pas dans des expériences témoins en l'absence de glucose.

3. On ne constate aucune différence dans la teneur en diverses fractions de phosphates: P minéral (y compris P de la créatine) P hydrolysable en 7 min, en 15 min, en 180 min et P total dans les diaphragmes de rats normaux ou de rats ayant reçu de l'adrénaline.

4. Il n'existe aucune différence notable dans la teneur en glycogène initial des diaphragmes d'animaux traités ou non. La présence de glucose au cours de l'incubation anaérobie semble inhiber

la glycogénolyse dans le diaphragme des animaux normaux. Il apparaît que des différences quantitatives dans la nature du substrat (glycogène, hydrates de carbone phosphorylés) ne peuvent provoquer l'inhibition importante que l'on observe dans la glycolyse du diaphragme d'animaux traités par l'adrénaline, par rapport à ce que donne le diaphragme des animaux témoins, en présence de glucose. Cette inhibition s'exerce uniquement sur le métabolisme du glucose ajouté.

5. Lorsque l'on utilise comme substrat de l'hexosediphosphate, on n'observe aucune inhibition de la glycolyse anaérobie du diaphragme des animaux traités à l'adrénaline. Ceci conduit à penser que l'action inhibitrice vis-à-vis du glucose s'exerce antérieurement à la réaction zymohexase dans la chaîne des réactions de la glycolyse.

6. Aucune inhibition directe de l'utilisation du glucose du diaphragme sous l'influence de l'adrénaline ajoutée *in vitro* n'a pu être observée.

#### ZUSAMMENFASSUNG

1. Die vorliegende Arbeit wurde mit der Absicht unternommen, um zu untersuchen ob die Zunahme des Blutzuckers durch Adrenalin wenigstens teilweise durch Hemmung der Glukoseaufnahme durch die Gewebe verursacht sein könnte; genauer ausgedrückt, ob die Hexokinaserreaktion, die die Geschwindigkeit der Glukoseaufnahme aus dem Blut durch die Gewebe bestimmt, direkt oder indirekt nach Injektion von Adrenalin gestört wäre.

2. Eine deutliche Hemmung der anaeroben Glykolyse des Zwerchfells trat bei Ratten auf, die 40–70 Minuten vorher Adrenalininjektionen (1 mg/kg subkutan) erhalten hatten. Diese Hemmung beträgt ungefähr 30–36%. Die manometrischen Resultate konnten durch chemische Milchsäurebestimmungen bestätigt werden. Die Hemmung tritt bei Blindproben bei Abwesenheit von Glukose nicht auf.

3. Bei der Bestimmung verschiedener Phosphatfraktionen wie anorganisches Ph (incl. Kreatinphosphat), Phosphat nach Hydrolyse während 7, 15 oder 180 Min, und Gesamtphosphat in den Zwerchfellen von Ratten, die Adrenalininjektionen erhalten hatten, konnten bei Vergleich mit normalen Kontrollen keine bedeutenden Unterschiede nachgewiesen werden.

4. Zwischen dem Glykogengehalt des Diaphragmas behandelter und normaler Tiere besteht im Beginn kein Unterschied von Bedeutung. Die Anwesenheit von Glukose während der anaeroben Inkubation scheint die Glykogenolyse im Zwerchfell normaler Tiere zu hemmen. Es hat den Anschein, dass die deutliche Hemmung, die bei Vergleich der Glykolyse des Zwerchfells von Tieren, die mit Adrenalin behandelt wurden, mit Kontrolltieren bei Anwesenheit von Glukose wahrgenommen wird, nicht durch quantitative Unterschiede der inneren Substrate, (Glykogen, phosphorylierte Kohlenhydrate) verursacht wird. Die Hemmung betrifft nur den Stoffwechsel zugefügter Glukose.

5. Wenn Hexosediphosphat als Substrat verwendet wird, wird keine Hemmung der Zwerchfellyglykolyse bei Tieren, die mit Adrenalin behandelt wurden, wahrgenommen. Dies führt zu der Annahme, dass die hemmende Wirkung gegenüber Glukose vor der Zymohexaserreaktion in der Reaktionskette der Glykolyse auftritt.

6. Eine direkte Hemmung des Glukoseverbrauchs des Zwerchfells unter Einfluss von *in vitro* zugefügtem Adrenalin kann nicht nachgewiesen werden.

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## RESEARCHES ON PLANT GROWTH REGULATORS

## XV. THE INFLUENCE OF FATTY ACIDS ON SOAPCOACERVATES

by

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## I. INTRODUCTION

In the course of time indications became more and more numerous that on the outside of protoplasm there is a layer which regulates the permeability or rather the intrability of the protoplasm for dissolved substances. Especially the experiments of CHAMBERS and his collaborators really leave no possibility for other opinions. A solution of sodium chloride, when brought into the interior of an Amoeba is absolutely harmless, but on the outside of the cell the salt has a toxic effect. With a solution of calcium chloride it is exactly the other way about: an Amoeba, brought into such a solution, does not experience the slightest damage, but if the  $\text{CaCl}_2$ -solution is injected into the Amoeba an irreversible flocculation results (CHAMBERS AND REZNIKOFF, 1926). Therefore, the protoplasm of these Amoeba is evidently covered with a protective outer layer.

Indications of such a regulating protoplasmic membrane can also be found with other organisms. It does not seem too presumptuous to postulate the presence of a protoplasmic membrane in all living cells. This does not mean that this membrane might be observed with a microscope and it must certainly not be confused with the cell-walls formed by living protoplasm as e.g., the cellulose-wall of plant cells. The membrane is part of the protoplasm which only owes its particular properties to its position (the border cell/medium). It has often been observed that when the protoplasm is damaged (the contents of which are usually miscible with water) a new membrane is formed spontaneously. This can be traced with certain dyes, which diffuse freely through the protoplasm but are checked by the protoplasmic membrane (NAEGELI).

There is but little direct information about these protoplasmic membranes. As the membrane will determine the permeability of the cell, experiments on the permeability may give indications regarding the structure of the protoplasmic membrane.

It is a difficult task to select the most satisfactory solution from the many given on the question of permeability. In our opinion all theories on permeability may be reduced to two principles:

- a. The permeating substance is soluble in the protoplasmic membrane.
- b. The permeating substance passes through pores in the membrane.

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\* Researches on plant growth regulators XV.

\*\* Influence of organic compounds on oleate and phosphatide coacervates VIII,

The lipid theory (OVERTON) is centered around the first principle and the filter theory (M. TRAUBE, RUHLAND) ascribes the permeability to the second mechanism. It seems worth while to show that the many other theories do not differ in essentials from these two theories.

1. The micellar theory (NAEGELI-PFEFFER) is essentially a filter theory.

Here it is important that the pores can be changed by external influences. The real filter theory does not harbour this possibility.

2. In our opinion the adsorption theory (J. TRAUBE) cannot be considered to be a permeability theory *sensu stricto*. Usually two factors that play a part in the permeating of substances are involved, *viz.* the permeability through the protoplasmic membrane and the instigating force — *i.e.*, the difference in the concentration of the substance on both sides of the membrane. If a compound is adsorbed at the protoplasmic surface it need not be concluded that the permeability for that compound is abnormally high, only that the instigating force is greater (at least if the solubility of the substance in the protoplasm is larger than in water). For a positive adsorption means a higher concentration and therefore a seemingly greater permeability. TRAUBE himself points out that the adsorption theory is essentially a support of the lipid theory and that it does not exist by itself.

3. Electrostatic influences — to which MICHAELIS draws the attention — can increase or decrease the permeability (depending upon whether the compound and the membrane have an opposite or a similar electric charge). In this case we must speak of influences upon the resistance.

4. A consideration of the so-called colloid-chemical theory of HANSTEEN CRANNER immediately shows that here we have to do with a variation of the lipid theory. HANSTEEN CRANNER only indicates that the membrane would consist of two phases: phosphatides soluble in water and insoluble phosphatides.

5. Besides these there are a few theories that try to combine the two principles. CLOWES compares the protoplasmic membrane with an emulsified colloid. Under the influence of certain electrolytes it is possible for an emulsion of oil in water to change into one of water in oil. According to CLOWES something similar would also take place in the protoplasmic membrane.

According to the mosaic theory (NATHANSON) the protoplasmic membrane is composed of two kinds of structures. The first will follow principle *a*, the other principle *b*. In a more modern setting this theory is called the lipid-filter theory (COLLANDER).

Reviewing these possibilities we can combine them all in a few rules:

1. Some molecules permeate as one would expect according to their solubility in lipids; while the permeation of other molecules is mainly determined by their size.

2. The biological objects are very different, all possible forms between principles *a* and *b* do exist.

It is evident that now our ideas concerning the protoplasmic membrane must fit in with these data. Hence we may conclude that a membrane of a thick lipid layer (thickness of many molecules), as well as a membrane without lipid, are not very probable. In the first case substances that are insoluble in lipid (even water) would not be able to permeate, in the second case one would not understand why non-polar substances permeate so quickly.

Then it appears that in molecular dimensions (a mono-, di-, or paucimolecular layer of lipid molecules) the principles *a* and *b* can be united without the necessity of

accepting a heterogeneous structure of the protoplasmic membrane as in the mosaic theory. In many cases it seems admissible that besides lipoids there are also proteins in the membrane. As there is little reason for these substances to accumulate on the border cell/medium under influence of the boundary tension, another reason must be found for this. We here think of complex relations (BUNGENBERG DE JONG, 1938, 1936): electrostatic attraction forces which are probably the strongest stabilising factors of the membrane. In connection with these considerations especially amphoteric phosphatides must be assumed to occur as constituents of the protoplasmic membrane (BUNGENBERG DE JONG AND BONNER, 1935). Fig. 1 gives a very schematic picture of a membrane, conceived in this manner.

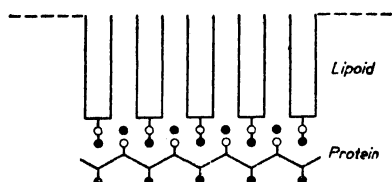


Fig. 1. Diagram of the structure of the protoplasmic membrane (the black spots represent positive groups, open circles are groups with a negative charge). This membrane is stable because of the strong complex relations in the polar part of the membrane. The component parts are: lecithin, protein and a cation (usually Ca).

Here we must note that the most important points of this theory of the protoplasmic membrane — which we should like to call the complex theory, because the complex-relations play such an important part — are the following:

1. The regulating part proper of the protoplasmic membrane with respect to uncharged molecules consists of hydrocarbon chains in positions parallel to each other (Fig. 1, upper part).

1. The regulating part proper of the protoplasmic membrane with respect to uncharged molecules consists of hydrocarbon chains in positions parallel to each other (Fig. 1, upper part).

Already some time ago BUNGENBERG DE JONG AND SAUBERT (1937) put forward the idea that in this membrane cholesterol would be present as a "condensing substance", e.g., in the way as shown in Fig. 2.

This diagram shows how the filter theory and the lipid theory are mutually compatible in molecular dimensions: place *a* represents a pore in the lipid-membrane. Care must be taken not to consider these diagrams from a static point of view only. The thermal movement of the paraffin chains will give the membrane an ever changing aspect. The chance that at some given moment a pore of small diameter will occur is great (even in a quite homogeneous membrane), and though the chance for wider pores decreases continuously, it does remain. This seems to be a plausible explanation for the fact that with an enlargement of the molecular volume of the permeating substance, the permeability for that substance decreases considerably. We do not consider it necessary to presume that there are many small pores, few large and very few very large pores. If the protoplasmic membrane is looked upon as a dynamic system then a homogeneous molecular layer of lipoids must also show this effect. It is evident that the permeability is very strongly affected by the nature of the paraffin chains (many or few unsaturated chains) and the amount and nature of the condensing substance (e.g., cholesterol).

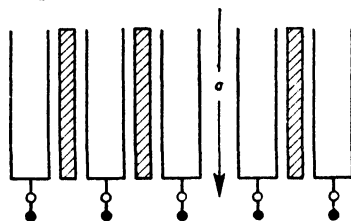


Fig. 2. Cholesterol can be present as a condensing factor between lecithin-molecules. Whenever the cholesterol is missing we have to do with a "pore" in the membrane (a). According to BUNGENBERG DE JONG AND SAUBERT, 1937.

2. The permeation of charged compounds (*viz.*, ions) will greatly depend on the momentary situation of the electric system of the membrane (Fig. 1, lower part).

WINKLER's experiments (1940) on the membrane of the pig's erythrocytes give

fine support to the idea that the membrane is a tricomplex system. WINKLER is able to conclude from the ionic spectrum of the erythrocytes that phosphatides play an important part. The membrane proves to be a tricomplex of phosphatide, stromatin and a cation. It will be possible to replace the cation in the protoplasmic membrane by another cation, which may give rise to great changes in the permeability (BOOIJ, 1940).

Of course our diagram (Fig. 1) is very schematic. It is not impossible that the phosphatide molecules are turned towards the interior or that there are several layers of parallelly arranged molecules present. It is of special interest, however, that the complex relations are of vital importance for the structure of the membrane. Without Coulomb forces between the polar groups of the membrane components it is impossible to imagine how on the boundary protoplasm/medium a layer of lipid molecules in parallel arrangement would be formed.

Certain properties of the protoplasmic membrane can be studied on coacervates of lecithin (BUNGENBERG DE JONG, 1937). But such coacervates are rather unmanageable, and if only the properties of the non-polar part of the membrane need be studied then one can make very good use of soap coacervates, since very probably the molecules in concentrated soap solutions are arranged in a parallel manner (HESS, 1941; STAUFF, 1941). Therefore this model will be especially useful for examining non-electrolytes or molecules with a large non-polar part. Such an investigation was made by BUNGENBERG DE JONG and collaborators (1938). In this regard it is important that the organic non-electrolytes — as to their reaction on oleate coacervates — can be placed in a certain sequence and that practically the same sequence is encountered in tracing the inhibiting influence of these compounds upon the germination of pollen of sweet pea (BOOIJ, 1940).

The natural and synthetic compounds acting as plant growth substances all consist of molecules with a considerable non-polar part. Besides this non-polar part they also contain a carboxyl group. VELDSTRA (1944) deduced from experiments on the relations between the structure and the activity of the plant growth substances that compounds of this structure may possibly influence the permeability of plant cells, in that sense that they would further the permeability by a turgescence or opening influence on the membrane at a low concentration (physiologically stimulating), whereas they would probably have a condensing effect in higher concentrations (physiologically inhibiting).

To test this hypothesis experiments were made regarding the influence of plant growth substances on systems which, to a certain extent, can be considered to be models of the protoplasmic membrane. This was done firstly by examining the interaction between the compounds mentioned and mono-molecular layers of lecithin (HAVINGA, VELDSTRA, 1948) and secondly, as is described in this and in the following paper, by examining the influence of growth substances on coacervates, especially on oleate coacervates. According to the hypothesis a pronounced interaction with the lecithin film and an opening effect on the above-mentioned coacervate-systems might be expected. Of course we must not forget that these are only models and that the real relations are far more complicated. All the same, clear indications can be obtained by these experiments.

Preliminary experiments by one of us (BUNGENBERG DE JONG) showed that indeed  $\alpha$ -naphthaleneacetic acid has a turgescence action on soap coacervates, as the coacervation is annulled by adding a small quantity of  $\alpha$ -naphthaleneacetic acid. The  $p_H$  of



these soap coacervates is high, so in this case the anion, and not the undissociated molecule, is active\*.

Many compounds had already been examined in connection with their action on oleate coacervates (BUNGENBERG DE JONG *et al.*, 1938; ROSENTHAL, 1939), but practically all these belonged to the non-electrolytes.

Before extending our experiments to the synthetic plant growth substances, we thought it would be worth while to examine previously more simple anions. For this we chose the homologous series of normal fatty acids. When the action of this series is known, the effect of the growth substances can be compared with it.

## 2. EXPERIMENTS WITH FATTY ACIDS (ANIONS)

BUNGENBERG DE JONG *et al.* (1938) examined a large number of organic compounds as to their influence on oleate coacervates and were able to deduce the following general rules. We must, however, emphasize the fact that these rules have only been found for non-electrolytes.

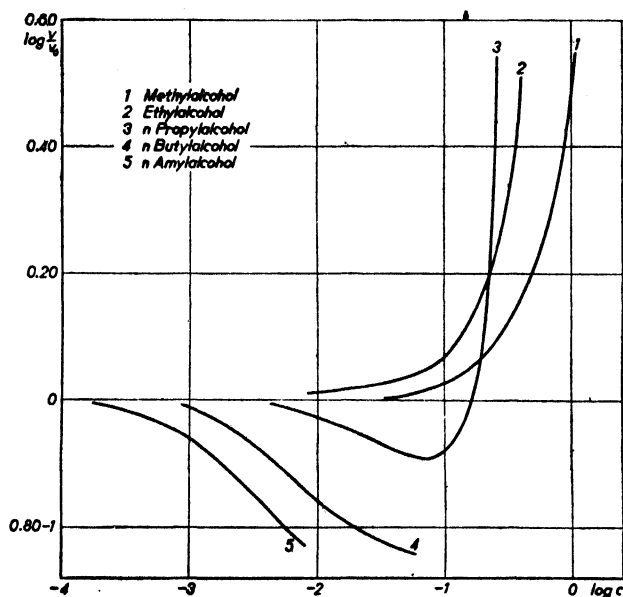


Fig. 3. Action of the normal alcohols on the volume of an oleate coacervate (BUNGENBERG DE JONG, BOOIJ AND SAUBERT, 1937).

1. In a homologous series of compounds with a polar group the minimal concentration displaying activity decreases with an increasing number of carbon atoms.

2. In such a series the reaction is generally inverted at a given chain-length, the lower terms having a turgescence, the higher a condensing effect. Fig. 3 shows this for the series of the normal alcohols.

\* Botanical experiments gave rise to the conviction that only undissociated molecules would be physiologically active (cf. STRUGGER, 1932; BONNER, 1938 and VAN SANTEN, 1940). In one of the following articles we shall discuss this contradiction more in detail.

3. The influence on oleate coacervates of compounds with a hydrophilic group is the result of the antagonistic action of two factors:

- a. a condensing influence of the hydrocarbon chain,
- b. an opening influence of the polar group.

4. The condensing effect of the hydrocarbon chain increases with its length. With an equal number of carbon atoms the influence decreases on branching of the chain, on ring-closure and on the substitution of a saturated six-membered ring by an aromatic nucleus.

5. Introduction of a halogen into an aliphatic chain results in a stronger condensing effect.

6. The OH-, ether-, ketone-, ester-oxygen-groups may be mentioned as promoting a turgescient effect.

7. By mutual comparison of the action of polar groups it appears that with equal numbers of carbon atoms *e.g.*, urethanes have a stronger effect than primary alcohols and that ketones act more strongly than secondary alcohols.

Resuming it may be concluded that the action of a compound is determined by the structure of the hydrocarbon chain (number and distribution of the carbon atoms and their type of binding) and by the nature, number and location of the polar groups.

The experimental method was as follows (BUNGENBERG DE JONG *et al.*, 1937): A gradually increasing quantity of KCl is added to an oleate solution and as a result two

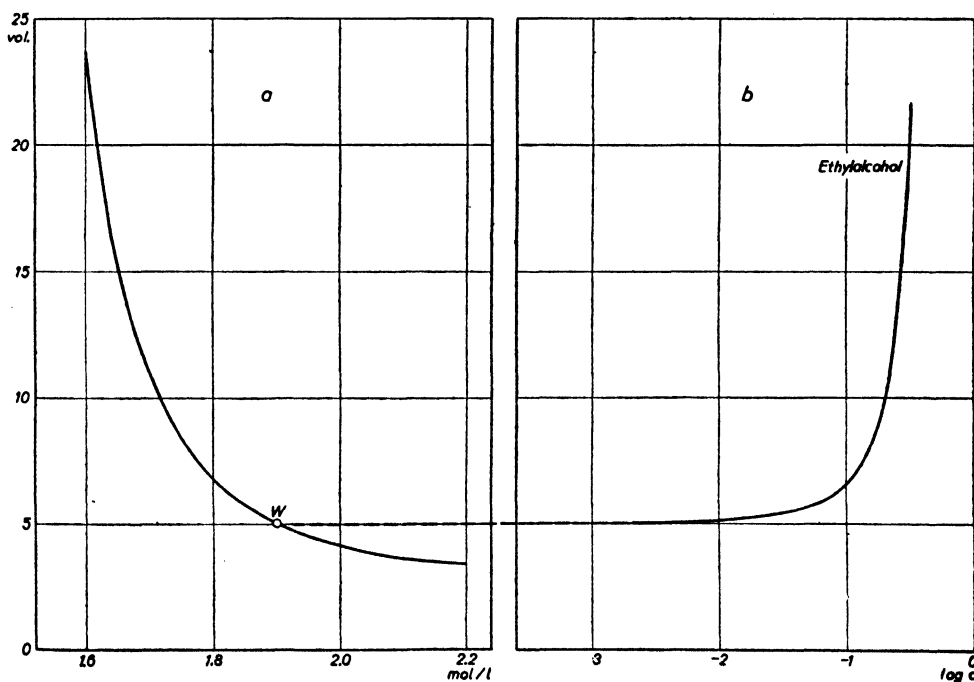


Fig. 4. Experimental method of BUNGENBERG DE JONG *et al.*, concerning the influence of different compounds on oleate coacervates.

a. KCl causes the formation of a coacervate in an oleate solution, the volume of which decreases as the concentration of KCl increases.

b. On this curve we choose a convenient working-point (w) and add (with a constant KCl concentration) increasing quantities of the compound to be examined (*e.g.*, ethyl-alcohol).

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liquid phases begin to separate at a certain concentration. The top layer contains practically all the soap. This layer becomes thinner as the concentration of KCl increases (see Fig. 4a).

We now choose a convenient working-point on this curve and add, keeping the KCl concentration constant, an increasing quantity of the compound to be examined (*e.g.*, ethylalcohol, see Fig. 4b). If the top layer (which is relatively rich in colloid) increases in volume—meaning that this layer gradually takes up more and more water—we have to do with a turgescence effect. The influence of a condensing substance will appear by a decrease of the coacervate layer.

This method is not suitable for examining the fatty acid anions. As is known, the solubility of soap rapidly becomes low. Most fatty acids, however, are well soluble in an oleate-solution to which an excess of KOH has been added. We can then compare the KCl curve before and after the addition of the fatty acid and the shifting of the curve tells us something about the action of the added substance.

So our method takes the following form: A 2% solution of sodium oleate is prepared (with a strongly sensitised soap it is advisable to add some KOH, see BUNGENBERG DE

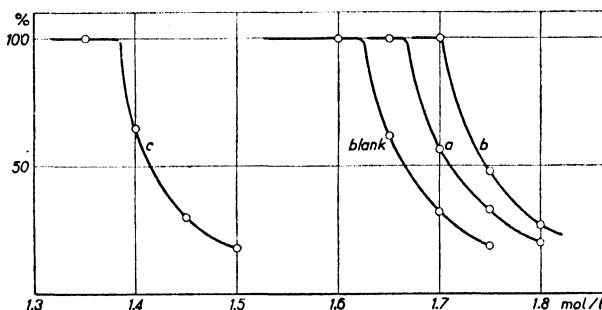


Fig. 5. After determining the KCl-curve for the separation of the oleate (blank) the influence of palmitate is measured ( $a = 0.625$  m mol;  $b = 1.25$  m mol). The graph shows the volume of the coacervate expressed as per cent of the volume of the whole liquid. The shifting of the KCl-line (taken at 50%) is a measure for the turgescence effect of the added compound. For comparison the influence of a condensing substance is also given ( $c = \text{benzylalcohol}$ , 5 m mol).

JONG *et al.*). To 500 ml of this solution are added 50 ml of 2 N KOH (this serves as a blank). The desired quantity of fatty is weighed and to this *e.g.*, 100 ml of the blank are added. From this solution, with the aid of the blank, different solutions are prepared, so that a series of oleate solutions is obtained in which the added compound is present in different concentrations. In wide test-tubes the following mixtures are made, which are shaken thoroughly:

5 ml of oleate (with or without added substance)  
 x ml of 3.8 N KCl  
 (14 - x) ml of  $\text{H}_2\text{O}$

The test-tubes are placed in a thermostat\* and the next day the height of the coacervate layer is read. We plot the percentage of the layer with respect to the total column against the KCl concentration (Fig. 5).

\* When we started these experiments it was not yet possible (on account of gas rationing) to work at a constant temperature. We therefore performed the experiments at room-temperature (which generally varied between  $12^\circ$  and  $16^\circ\text{C}$ ) and to avoid sudden changes of temperature the tubes were placed in a large volume of water. The influence of the temperature on coacervation is, however, rather appreciable and as soon as it was possible we worked in a thermostat.

The shifting of the KCl-curve to higher concentrations means that the added palmitate ion has a turgescence effect. Evidently more KCl is necessary to attain the same degree of coacervation. For comparison the curve for the addition of a condensing substance (benzylalcohol) is also given.

In this way the homologous series of normal fatty acids\* was examined (see Table I).

TABLE I  
CONCENTRATION OF ADDED FATTY ACID ANION IN MMOL

Number of C-atoms	0.15 <sup>5</sup>	0.31	0.62 <sup>5</sup>	1.25	2.5
8	—	—	—	0.05 <sup>5</sup>	0.12 <sup>5</sup>
9	—	—	—	0.17 <sup>5</sup>	0.41
10	—	—	—	0.39	0.91
11	—	—	—	0.44	0.90
12	—	—	—	0.37 <sup>5</sup>	0.73
13	—	—	—	0.27 <sup>5</sup>	0.51
14	—	—	—	0.18	0.32
15	—	—	—	0.09	0.17
16	—	—	0.04 <sup>5</sup>	0.08	0.12
18	—	—	0.06 <sup>5</sup>	0.11 <sup>5</sup>	0.11
20	0.05 <sup>5</sup>	0.10	0.20 <sup>5</sup>	0.22 <sup>5</sup>	—
22	0.06	0.10	0.21	0.22 <sup>5</sup>	—

Shifting of the KCl-line (in normality) under influence of different fatty acid anions (temperature 12–16° C). The substrate is an oleate coacervate.

On examining the higher terms difficulties are encountered owing to the insolubility of these soaps, as shown in Fig. 6. Generally the shifting of the KCl-curve in low concentrations (the point at a height of 50% is considered) is proportional to the concentration of the added compound.

In order to compare the effects of the different fatty acid anions we must of course remain below the concentration at which the curves begin to flatten. Hence we plot the shifting of the KCl concentration, caused by adding 0.5 m mol of anion, against the number of carbonatoms (see Fig. 7).

The activity-curve of the homologous series of the fatty acid anions with 8 to 22 C-atoms shows an unexpected and remarkable course. At first sight there does not seem to be any reason whatsoever that the turgescence effect should first increase with the lengthening of the chain, then decrease and finally increase again. What we expected theoretically — owing to the simple constitution of the added compounds and of the oleate — was an effect (either turgescence or condensing), becoming more marked with the lengthening

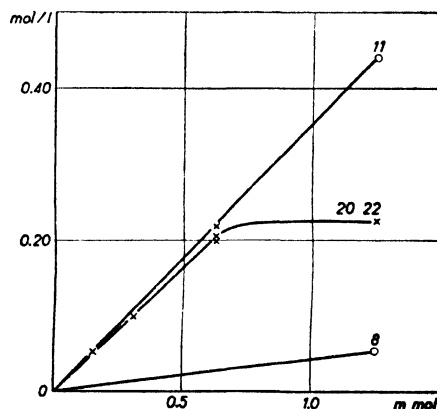


Fig. 6. The shifting of the KCl-curve (in mol/l) for the lower members of the homologous series of fatty acid anions is practically proportional to the concentration (compare undecanoate(11) and caprylate(8)). With the higher members trouble is encountered in the insolubility above a certain concentration (see arachidate(20) and behenate(22)).

\* We are very much indebted to Prof. Dr P. E. VERKADE, Delft, who was so kind as to place a large number of fatty acids of high purity at our disposal.

of the carbon chains. From former experiments (BUNGENBERG DE JONG *et al.*, 1937) we already knew that the long-chain alcohols have a strong condensing effect. Replacing the OH-group by a carboxyl-group (in a dissociated state) causes a strong turgescent effect. Obviously it is our first task to explain the remarkable activity curve of the fatty acid anions.

### 3. THEORETICAL BACKGROUND OF THE EXPERIMENTS

Trying to explain Fig. 7 we come to consider the following possibilities:

1. Though not easily explainable on theoretical grounds, it might be possible that at

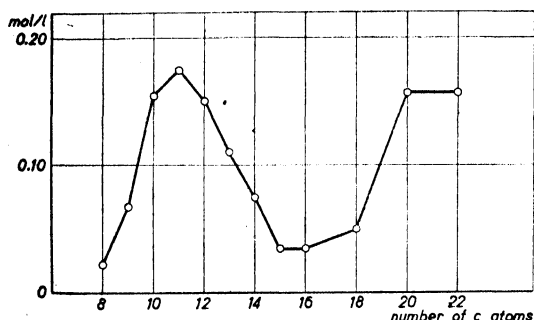


Fig. 7. Shifting of the KCl-curve (in mol/l) under influence of 0.5 m mol. of added fatty acid anion (the substrate is an oleate coacervate, temperature 12–16° C). The influence is evidently strongly dependent on the number of carbon atoms of the added compound

certain lengths of the hydrocarbon chains a stronger effect occurs, expressing itself in a periodicity. Then we would conclude from Fig. 7 that in the neighbourhood of valeric acid there will be another minimum. Under the conditions sketched in Fig. 7 the lower terms show no effect whatsoever in low concentrations so that in this case another method ought to be applied.

It would certainly also be worth while to examine acids longer than behenic acid, but few of these acids are as yet known and obtainable in the pure state.

2. It is known that many soaps are comparatively poorly soluble and crystallise from their solutions. The solubility is much greater in oleate solution than in water. If we now presume that the higher members of the homologous series form small crystalline micells when KCl is added, independent of the oleate coacervate, then we can understand the decrease of the activity of these higher terms. These micells must, however, be very small indeed, as the coacervates are absolutely clear. Moreover the increase of activity of arachidic and behenic acid remains incomprehensible. In any case an increase of the temperature should have a considerable influence because it checks the forming of crystalline micells.

3. There are indications that in soap solutions micells are present, in which the soap molecules (or ions) are arranged in a parallel manner (HESS *et al.*, 1941). It does not seem improbable that there is also a similar arrangement in soap coacervates. Considering such an oleate coacervate, in which the oleate molecules are somehow arranged parallel to one another, it does not matter for the experiment whether or not we add a small extra quantity of oleate. Molecules of a different length (shorter as well as longer ones) do not fit into the orderly micell; the order is disturbed and we observe this as the "turgescent effect" of these non-fitting molecules. That is how this conception can explain the minimum at 15 to 16 C-atoms. We must remember that the effective length of the hydrocarbon chain of the oleate molecule is certainly less than 18 C-atoms because oleic acid possesses the *cis*-configuration.

First of all we turn our attention to the lower members of the homologous series. As already mentioned these anions show no activity in low concentrations. Only with

caproate can we demonstrate a slight turgescence effect with the above-mentioned method. If we want to test the lower terms we will have to change our method. The method to be followed is clear, instead of KCl we use KOH as a means of causing the coacervation. We also do not dissolve the acid to be examined in the oleate solution, but in 5 N KOH.

Mixtures, treated in the same way as in the previous method, (p. 248), are prepared as follows:

- 5 ml of oleate 2%
- 5 ml of a solution of fatty acid  
in 5 N KOH
- x ml 5 N KOH
- (10 - x) ml of H<sub>2</sub>O

The lower terms of the series then appear not to have a turgescence, but a condensing effect (Fig. 8).

Only formic acid — a compound which differs in many respects from the other members — has a very weak turgescence effect. The lower fatty acid anions only being active at high concentrations, it seems possible that this effect has an origin (salt effect), different from the action of the higher terms. We must of course still verify whether the higher terms show the same reactions in the KOH-method as in the KCl-method. This indeed proves to be the case; the maximum of the activity-curve remains present at undecanoic acid. The shape of the curve is absolutely comparable with that of Fig. 7. There is no question of a minimum in effect at valeric acid — as might have been expected should a certain periodicity occur in the homologous series. Therefore we can now drop the idea of a periodicity that cannot hold good theoretically and practically.

The second possibility was that the higher terms of the series of fatty acid anions should form independent micells. A drawback of this idea has already been mentioned: the coacervate layers remain quite clear, so that we might presume that the formed micells are very small. And moreover the strong turgescence effect of arachidate and behenate cannot be explained by this supposition. On second thoughts we can already deduce from Fig. 6 an explanation of the problem of independent micells. As long as there are no independent micells the effects of the added compound must be more or less proportional to the concentration. The moment independent micells are formed this proportion disappears and the curve will run parallel to the abscissa. The curves for arachidate and behenate indeed show this course. Therefore we must compare the effects of the homologous series at low concentrations.

The formation of independent crystalline micells is, from its very nature, very dependent on the temperature. An increase of temperature to 40° proves to have practically no effect on the shape of the activity curve of Fig. 7, however. Therefore we must conclude that this curve is indeed real and that formation of micells does not play an

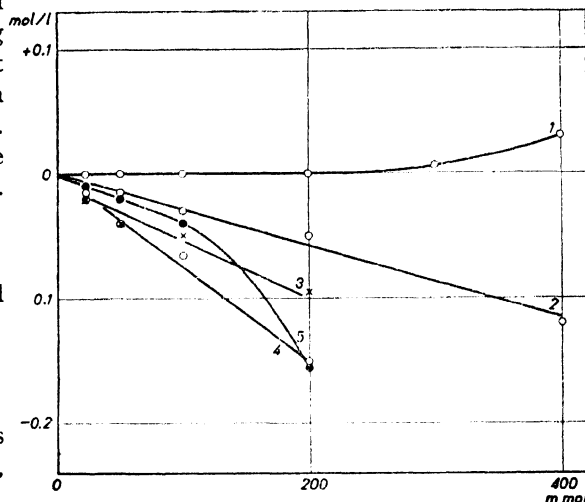


Fig. 8. The lower terms of the series of fatty acid anions only act in high concentrations. Formate(1) opens weakly, the other salts: acetate(2), propionate(3), butyrate(4) and valerate(5) condense slightly.

important rôle. The curves for arachidate and behenate remain linear over a longer stretch (Fig. 9) because at a higher temperature the formation of independent micells takes place with a higher concentration.

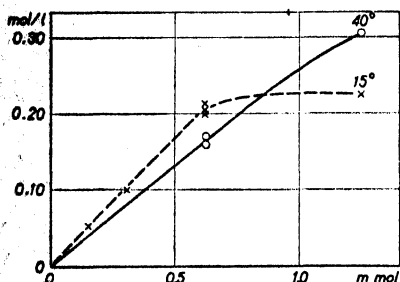


Fig. 9. The solubility of arachidate and behenate is larger at a higher temperature ( $40^{\circ}\text{C}$ ) than at a temperature of  $15^{\circ}\text{C}$ ; in the first case the effects of these compounds remain proportional to the concentration for a longer period.

be found inside the micells ( $C_M > C_E$ ). As they are not taken up by the micells, the lower members must have hardly any influence on the order of the micells. The area between caproate and undecanoate would be the transition range between the lower and the higher members. The effect increases with a lengthening of the carbon chain because more and more of the compound is adsorbed by the oleate micells.

We can verify this idea by making experiments at different oleate concentrations. We examine, by means of the method described, the shifting of the KCl-curve under influence of stearate, starting from 1%, 2% and 4% standard solutions of sodium oleate. Table II shows the shiftings found.

TABLE II

Concentration of stearate	Shifting of the KCl-curve (in mol/l) with oleate of:		
	1%	2%	4%
0.31 m mol	0.06 N	0.02 <sup>5</sup> N	0.01 N
0.62 <sup>5</sup> m mol	0.10 N	0.05 N	0.02 N
1.25 m mol	0.15 <sup>5</sup> N	0.09 N	0.05 N

If we assume the experimental error not to exceed 0.01 N, we can draw the following conclusions. The shifting of the KCl-curve (turbescent effect) is proportional to the concentration of the stearate. The only deviation from this rule is found at 1.25 m mol. stearate when the oleate concentration is low. This must be ascribed to the fact that as the oleate concentration decreases, the solubility of the stearate also decreases. The most important point, however, is that the effect is inversely proportional to the oleate concentration. If the amount of oleate is twice as little, the effect of the stearate is twice as large. We can now find graphically which stearate concentrations are necessary to cause a shifting of 0.05 N KCl. If we plot these figures against the oleate concentration of the standard solutions we see immediately that the curve goes through zero (Fig. 10). This means that all the added stearate has been adsorbed into the oleate micells.

In the same way we examine nonanoate. Table III shows the shifting of the KCl-curve under influence of nonanoate at different oleate concentrations.

TABLE III

Concentration of nonanoate	Shifting of the KCl-curve (in mol/l) for oleate solutions of:		
	1 %	2 %	4 %
0.31 m mol	0.04 N	0.03 <sup>5</sup> N	0.03 N
0.62 <sup>5</sup> m mol	0.09 N	0.08 <sup>5</sup> N	0.07 N
1.25 m mol	0.19 N	0.17 <sup>5</sup> N	0.15 N

It is remarkable that here we find no proportion whatsoever between the effect and the inverse of the oleate concentration. The calculation of the equilibrium becomes complicated because the blank lines for 1, 2 and 4 % standard solutions of oleate run differently. Therefore it is better to compare the shifting of  $\log C_{KCl}$  under influence of the added nonanoate (Table IV).

TABLE IV

Concentration of nonanoate	Shifting of $\log C_{KCl}$ for oleate solutions of:		
	1 %	2 %	4 %
0.31 m mol	.0113	.0097	.0079
0.62 <sup>5</sup> m mol	.0237	.0244 (?)	.0182
1.25 m mol	.0508	.0475	.0382

If we now determine graphically the nonanoate concentrations that cause a shifting

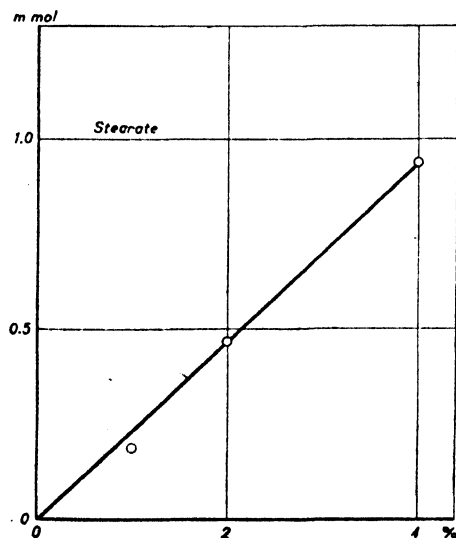


Fig. 10. The quantity of stearate required to cause a shifting in the KCl-curve of 0.05 mol/l (plotted along the abscissa) varies for different concentrations of the oleate solution (standard: 1, 2 and 4 %). The line connecting these points goes through zero, so evidently all the stearate is adsorbed into the oleate micells.

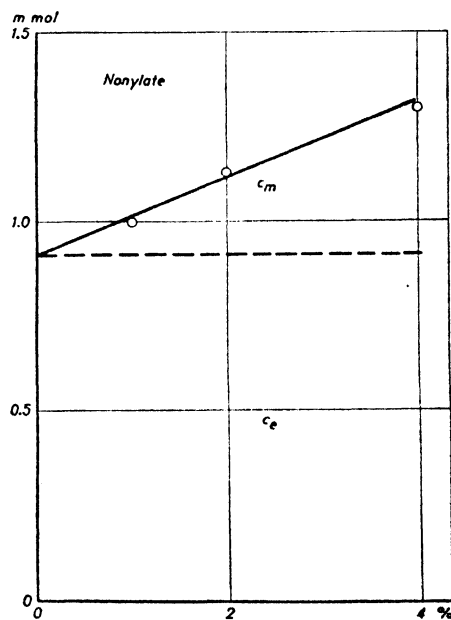


Fig. 11. The situation for nonanoate differs from that for stearate. The line cuts the ordinate at 0.91 m mol of nonanoate so that this must be the equilibrium-concentration of this compound (see also Fig. 10).



of 0.04 in  $\log C_{KCl}$  we find a line that does not run through zero (Fig. 11). For nonanoate there is evidently a high equilibrium-concentration ( $C_E$ ). The quantity ( $C_M$ ) adsorbed by the micells is of course proportional to the number of micells available (*i.e.* the concentration of the oleate).

In a certain case — a shifting of the  $\log C_{KCl}$  of 0.04 and for an oleate solution of 2% —  $C_M$  is 19% of the quantity of the added nonanoate.

The same experiment, performed with undecanoate, shows that for this compound the quantity adsorbed by the oleate-micells is much greater (Fig. 12). If we take the same circumstances as in the case of nonanoate — shifting of  $\log C_{KCl}$  by 0.04 and for an oleate standard solution of 2% — then  $C_M$  is 83%.

An experiment with behenate affirms that of the higher terms 100% is adsorbed into the oleate micells (Fig. 13).

It is evident that the figures for the lower members are only of a comparative value; they only apply to certain conditions. Nevertheless, the conclusion which may be drawn from these experiments is clear: the lower members of the homologous series have no effect or only a weak one in the lower concentrations, because they are not, or only slightly adsorbed into the oleate micells. It has already been explained why the curve for the adsorption (Fig. 13) does not correspond with the activity-curve (Fig. 7). It is of course logical to test this first for a stearate coacervate. In principle experiments with

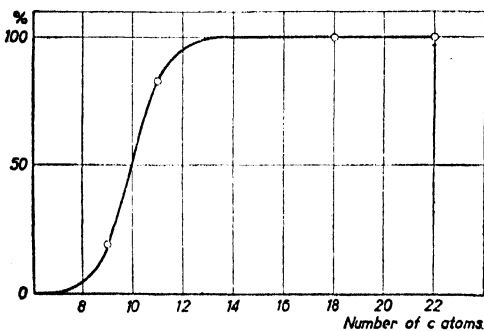


Fig. 13. We can deduce from Figs. 10, 11 and 12 which percentage of the added fatty acid anion is absorbed into the micells under certain circumstances (standard of the oleate solution = 2%, shifting in  $\log C_{KCl} = 0.04$ ).

An experiment on the influence of the same series of fatty acid anions on a substrate of a compound with a totally different structure (*viz.*, on a coacervate of desoxycholate) now becomes very interesting.

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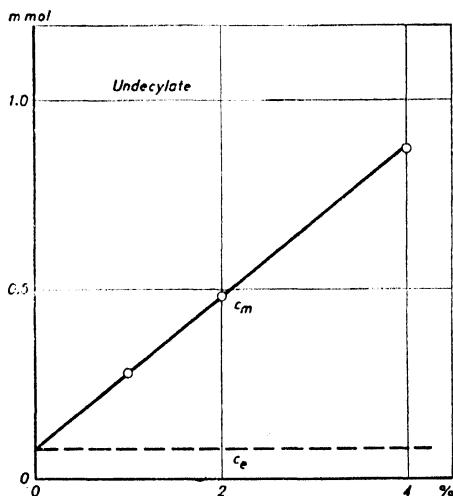


Fig. 12. For undecanoate the equilibrium concentration is lower than for nonanoate (compare Fig. 10 and 11).

stearate coacervates are completely comparable with those on oleate coacervates. The only important difference is that we use  $K_2CO_3$  instead of  $KCl$  and that the temperature must be chosen high ( $60^\circ C$ ). If our theory is right then the minimum in the activity-curve must be situated at 18 carbon atoms and the experiment confirms this prediction completely (see Table V and Fig. 14).

The depth of the minimum in the activity-curve depends on the degree of conformity between the substrate and the added compound. As a matter of fact the minimum is deepest when the added ion corresponds with the substrate (see, in Fig. 14, the effect of stearate on a stearate coacervate).

TABLE V

Number of C-atoms of fatty acid	Shifting of the $K_2CO_3$ curve in (mol/l) by fatty acid anions	
	Conc. 1.25 m mol	Conc. 2.50 m mol
6	0.00 <sup>5</sup>	0.01 <sup>5</sup>
8	0.08 <sup>5</sup>	0.26 <sup>5</sup>
9	0.24	0.59
10	0.30 <sup>5</sup>	0.64
11	0.28	0.55
12	0.21	0.42 <sup>5</sup>
13	0.16	0.30
14	0.08 <sup>5</sup>	0.17 <sup>5</sup>
15	0.03	0.06 <sup>5</sup>
16	0.00 <sup>5</sup>	0.01
18	- 0.01	- 0.02 <sup>5</sup>
20	0.09 <sup>5</sup>	0.18
22	0.07 <sup>5</sup>	0.11 <sup>5</sup>

Shifting of the  $K_2CO_3$ -curve (in mol/l) under the influence of fatty acid anions of different length (temp. 60° C). The substrate is a stearate coacervate.

If 1 ml of a 10% solution of desoxycholate is mixed with a concentrated solution of NaCl or KOH a coacervate is formed. The water content of this coacervate, however, is low, so that volume measurements do not offer many possibilities. With an increase of temperature the coacervate suddenly disappears and this criterion is useful in our experiments. We bring about 5 ml of the mixture to be measured into a wide test-tube, stirring the liquid with a thermometer. Besides this, glass beakers filled with water of  $\pm 60^\circ\text{C}$  and  $\pm 10^\circ\text{C}$  respectively are kept at hand. First of all we examine the influence of KOH on the temperature of separation. This point can be fixed with an accuracy of about  $0.2^\circ\text{C}$ , many times running. The mixtures are of the following composition:

1 ml of sodium desoxycholate 10%  
x ml of 5 N KOH  
(39 - x) ml of  $\text{H}_2\text{O}$

Upon increase of the KOH concentration the temperature of separation rises rapidly (Fig. 15).

We now choose a convenient working point on this curve (about  $51^\circ\text{C}$ ) and prepare a supply of a mixture that possesses this temperature of separation. From this supply 5 ml — at a temperature higher than  $51^\circ\text{C}$  of course — are brought by means of a pipette into wide test-tubes, which already contain the compounds to be examined. The substance is weighed by means of a torsion balance. The higher fatty acid anions

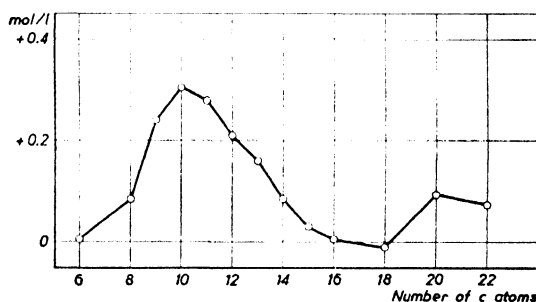


Fig. 14. The activity-curve of the fatty acid anions on a stearate coacervate shows a minimum at 18 carbon atoms, in contrast to an oleate coacervate for which this minimum is found at 15 to 16 C-atoms (see Fig. 7)

have such strong effects that this weighing-method cannot be used. In that case we dissolve a small quantity of fatty acid in 5 ml of ligroin. Of this solution we bring 0.25, 0.5 and 1 ml into the test-tubes with a pipette, and evaporate the solvent.

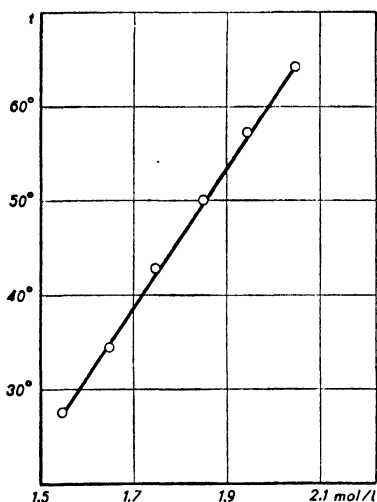


Fig. 15. Addition of KOH to a solution of desoxycholate causes a turbidity (coacervate). With higher concentrations of KOH the temperature at which this coacervate disappears is also higher.

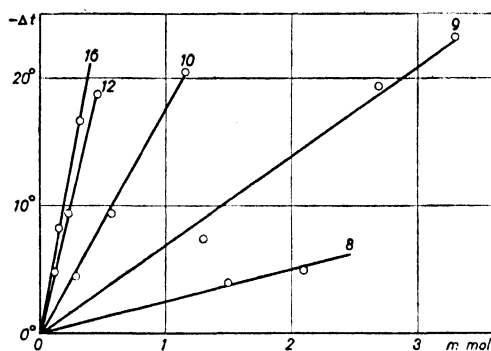


Fig. 16. Decline of the temperature of separation ( $-\Delta t$ ) of a desoxycholate coacervate under influence of fatty acid anions with a different number of C-atoms (8 = octanoate, 9 = nonanoate, etc.).

As in the oleate coacervate the fatty acid anions have a turgescent effect, which is observed by a decline of the temperature of separation. If we plot this decrease ( $-\Delta t$ ) against the concentration of the added substance the effect can be read immediately (Fig. 16).

If we now compare the lowering of the temperature caused by a certain concentration of fatty acid anions, the result is again an activity curve, but this time it does not show a definite minimum (Fig. 17). We do, however,

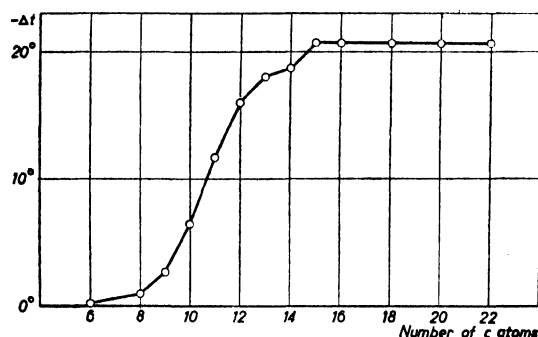


Fig. 17. Activity-curve of the fatty acid anions with a desoxycholate coacervate. There is no obvious minimum in the turgescent effect (as in Fig. 7 and 14).

the thickness of these micells is about equal to twice the length of the molecules of tridecanoic acid or of tetradecanoic acid, then we can expect that their ions will be slightly less active than the higher fatty acid anions.

We can also again determine the quantity of the added substance that is adsorbed

into the micells ( $C_M$ ). The method is the same as was applied with the oleate coacervates; we compare the effect of the added compounds at different desoxycholate concentrations. The resulting curve (Fig. 18) exhibits a great conformity with the activity-curve, although the irregularity at 13 and 14 carbon atoms has disappeared.

Attempts to determine the activity-curve with other substrates have failed. We tried to examine the influence of fatty acid anions on the separation of phenol and water, aniline and water, cresols and water. The results obtained are not reproducible, as these compounds are oxidised in air. Experiments with a coacervate of dehydrocholate also gave results which were too erratic to be of use.

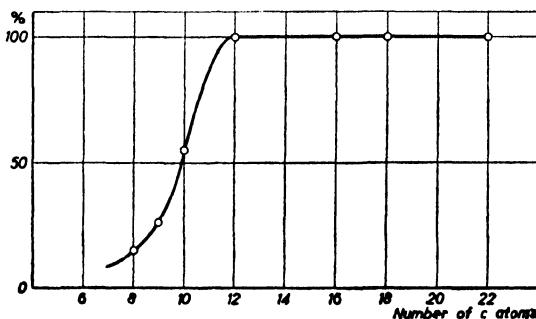


Fig. 18. Percentages of the added fatty acid anions that are adsorbed into the desoxycholate micelles.

### CONCLUSION

If we study the effect of a homologous series of fatty acid anions on an oleate coacervate, we observe a minimum in the activity-curve. Generally all higher fatty acid anions have a turgescence (opening) effect on such a coacervate, this in contrast to the higher alcohols. Particularly important in connection with the minimum in the activity-curve are the views on the conformity in structure of the substrate and the agent. If these are identical then practically no effect is to be expected (see *e.g.*, in Fig. 14 the effect of stearate on a stearate coacervate). If there is a great difference in length between the molecules of the agent and of the substrate, then the activity is high (*e.g.*, undecanoate as agent and an oleate coacervate as substrate, Fig. 7). Of course an agent can also be inactive because it is not adsorbed into the micells of the substrate. This is the case with the lower fatty acids (compare the rise in the curve in Fig. 7 and Fig. 13). In the desoxycholate molecule there is little conformity with the fatty acid anions. Therefore the activity curve (Fig. 17) only shows a slight irregularity, does not have a definite minimum and strongly resembles the curve representing the adsorption of the fatty acid anions into the micelles (Fig. 18).

It must not be considered improbable that such a turgescence effect may offer an explanation for certain physiological or pharmacological reactions. The remarkable feature is that one single molecule can disturb the order of a large number of molecules (see also SCHEIBE, 1939). We may reasonably presume that in many organisms there exists a certain order of lipophilic molecules. In this connection we may call to mind the protoplasmic membrane, the nerves, the grana; indeed, we may perhaps say that within organisms containing lipids a certain order of these molecules may be encountered (see for example the work of BEAR, PALMER AND SCHMITT, 1941).

Other compounds may then have a disturbing effect on organs that are characterized by such an order of molecules. It now becomes very interesting to examine more in detail the conditions for the occurrence of this disturbing activity. According to the starting-point of these investigations this problem will be studied first by trying to answer the question whether the action of the synthetic plant growth substances is

due to an effect on the protoplasmic membrane in the sense that the permeability for water (and for water-soluble compounds) increases. From the points of view given it follows that it is logical to attack this problem by examining the effects of growth substances and related compounds on oleate coacervates.

#### SUMMARY

1. The complex theory of permeability (BUNGENBERG DE JONG) affords a possibility to unite the lipid theory and the filter theory. If the protoplasmic membrane is conceived as a tricomplex system, composed of phosphatides, proteins and cations, the differences between these well-known theories vanish, when considered in molecular dimensions.

2. Oleate coacervates may serve very well as a model for the non-polar part of the protoplasmic membrane. The influence of many on this model organic compounds has been determined previously; these investigations were now extended by experiments on the influence of fatty acid anions.

3. The influence of fatty acid anions proved to be strongly dependent on the length of the carbon chain, very remarkable differences being found (Fig. 7).

4. In general the fatty acid anions demonstrate a turgescence effect (the water content, and so the volume, of the coacervate augments) at low concentrations. This influence becomes perceptible with six carbon atoms in the molecule and, in the homologous series, strongly increases up to the anion with eleven carbon atoms. After a minimal activity at fifteen C-atoms it rises again.

5. The first increase of activity can be explained by the distribution of the added fatty acid anions between the equilibrium liquid and the soap micells; with a growing number of C-atoms increasing amounts are adsorbed by these micells. On that occasion the added fatty acid anion exerts a disturbing action upon the orderly soap micells.

6. This disturbing action is minimal if the added fatty acid anion fits into the order of the oleate micells (in the case of 15 carbon atoms).

7. With another substrate (e.g. stearate coacervates) the minimum appears at a different point (with 18 C-atoms).

8. If there is but little similarity between the substrate and the added fatty acid anion (as in the case of sodium desoxycholate coacervate), a pronounced minimum is not observed any longer.

9. This obvious difference in activity of compounds from a homologous series, dependent on structural relations between "substrate" and "agent", may be of primary importance to the general problem of the influence of chemical structure on physiological action.

#### RÉSUMÉ

1. La théorie des complexes de la perméabilité (BUNGENBERG DE JONG) fournit une possibilité de combiner la théorie des lipides et la théorie du filtre. Si la membrane protoplasmique est en effet considérée comme un système tricomplexe, constitué par des phosphatides, des protéines et des cations, les différences entre les deux théories disparaissent si on se place à l'échelle des dimensions moléculaires.

2. Des coacervats d'oléate sont un bon modèle pour la partie non polaire de la membrane protoplasmique. L'influence de nombreuses substances organiques sur ce modèle a été déterminée précédemment. Ces recherches ont été étendues à l'influence des anions d'acides gras.

3. L'influence des anions d'acides gras s'est montrée sous la dépendance directe de la longueur de la chaîne carbonée (Fig. 7).

4. En général, les anions d'acides gras manifestent une action turgescence à des concentrations faibles. Cette action devient perceptible à partir de 6 atomes de carbones dans la molécule et, pour les homologues, s'accroît fortement jusqu'à 11 atomes de carbone. Après un minimum pour 15 atomes, cette action croît de nouveau.

5. Le premier accroissement d'activité peut être expliqué par la répartition des anions d'acide gras entre le liquide en équilibre et les micelles de savon. Lorsque le nombre d'atomes de carbone s'accroît, une quantité plus grande des anions d'acide gras est adsorbée par les micelles, ce qui provoque un désordre dans les micelles de savon.

6. Un tel désordre est réduit au minimum lorsque l'anion d'acide gras est tel qu'il puisse participer à l'ordre des micelles d'oléate (c'est le cas pour 15 atomes de carbone).

7. Avec un autre substrat (p. ex. des coacervates de stéarate), le minimum se manifeste pour un nombre d'atomes de carbone différent (18).

8. S'il n'existe que peu d'analogie entre le substrat et l'anion d'acide gras ajouté (comme dans le cas d'un coacervat de desoxycholate de sodium), on n'observe pratiquement plus de minimum.

9. Une différence aussi marquée dans l'activité de substances appartenant à des séries analogues, différences dépendant des relations de structure entre "substrat" et "agent", peut être d'importance fondamentale dans le problème général des relations existant entre la structure chimique et l'action physiologique.

*References p. 259.*

## ZUSAMMENFASSUNG

1. Die Komplextheorie der Permeabilität (BUNGENBERG DE JONG) ergibt die Möglichkeit, die Lipoid- und Filtertheorie zu vereinigen. Wenn die Protoplasmamembran als ein trikomplexes System aufgefasst wird, das aus Phosphatiden, Eiweiss und Kationen zusammengesetzt ist, fallen die Unterschiede zwischen obengenannten wohlbekannten Theorien weg, wenn man sie in molekularen Dimensionen betrachtet.

2. Oleatkoazervate können sehr gut als Modell für den unpolaren Teil der Protoplasmamembran dienen. Der Einfluss vieler organischer Verbindungen auf dieses Modell ist bereits früher bestimmt worden; diese Untersuchungen wurden jetzt durch Versuche über den Einfluss von Fettsäureanionen erweitert.

3. Hierbei wurde bewiesen, dass der Einfluss der Fettsäureanionen in starkem Masse von der Länge der Kohlenstoffkette abhängt, wobei sehr bemerkenswerte Unterschiede festgestellt wurden (Abb. 7).

4. Im allgemeinen zeigen die Fettsäureanionen bei niedrigen Konzentrationen einen Turgeszenzeffekt (der Wassergehalt, und dadurch ebenso das Volumen, des Koazervats nimmt zu). Dieser Einfluss wird bei sechs Kohlenstoffatomen im Molekül deutlich wahrnehmbar und steigt in der homologen Reihe erheblich bis zum Anion mit elf Kohlenstoffatomen. Nach einem Aktivitätsminimum bei fünfzehn Kohlenstoffatomen steigt er wieder.

5. Der erste Aktivitätsanstieg kann durch die Verteilung der zugefügten Fettsäureanionen zwischen der Gleichgewichtsflüssigkeit und den Seifenmicellen erklärt werden; bei steigender Anzahl C-Atome werden zunehmende Mengen von diesen Micellen adsorbiert. Hierbei übt das zugefügte Fettsäureanion eine Störwirkung auf die geordneten Seifenmicellen aus.

6. Diese Störwirkung ist minimal, wenn das zugefügte Fettsäureanion in die Ordnung der Oleatmicellen passt (im Falle von fünfzehn Kohlenstoffatomen).

7. Bei einem anderen Substrat (z.B. Stearatkoazervaten) tritt das Minimum bei einem anderen Punkt auf (bei 18 C-Atomen).

8. Wenn nur eine sehr geringe Ähnlichkeit zwischen dem Substrat und dem zugefügten Fettsäureanion besteht (wie im Falle des Natriumdesoxycholatkazervats), tritt ein ausgesprochenes Minimum nicht mehr auf.

9. Diese deutlichen Unterschiede in der Aktivität von Verbindungen aus einer homologen Reihe, die von strukturellen Beziehungen zwischen "Substrat" und "Agens" abhängen, sind möglicherweise von grösster Bedeutung für das allgemeine Problem des Einflusses der chemischen Struktur auf die physiologische Wirkung.

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## RESEARCHES ON PLANT GROWTH REGULATORS

XVI. THE EFFECT OF PLANT GROWTH SUBSTANCES ON  
COACERVATES

by

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## I. INTRODUCTION

On the whole one can form two different ideas concerning the mode of action of ergons in general and therefore also concerning compounds active as growth substances, which we will now consider more closely.

First of all we think of a function as a "carrier" in an enzymatically regulated process, as in the case of a number of vitamins of the B-group, which act as hydrogen-transporting coenzymes in oxidation-reduction chains, including a chemical reaction with the substrate.

As an alternative there is the possibility of an effect in which the part of the cell with which the interaction takes place ("receptor"), is influenced in a strictly physico-chemical sense and in which case there is no question of an ordinary chemical reaction (for a detailed discussion of these questions cf. VELDSTRA, 1947).

In preceding investigations concerning plant growth substances (VELDSTRA, 1944) the last-mentioned idea has been chosen as a starting hypothesis. This was done especially on account of the particular structure and configuration essential for growth substance activity in the compounds concerned. The physico-chemical influence was supposed to be exerted upon the protoplasmic membrane in such a manner that the growth substances, in physiologically active concentrations, would exert a turgescence effect on this membrane and that as a result of this the possibilities for transport of water and substances dissolved therein would increase. By this means the cell-elongation might then also be influenced.

The work of THIMANN AND SCHNEIDER (1938) and that of BUNGENBERG DE JONG *et al.* (1938) was considered to contain an important argument in favour of this conception. The former investigators studied the influence of neutral salts on the growth of *Avena coleoptiles* at a maximal active auxin concentration.

Growth is stimulated and the cations exert their action in the order:  $\text{Ca} < \text{Li} < \text{Na} < \text{K}$ . This is the same series as that in which these cations appear in the ionic spectrum of lecithin (TEUNISSEN, 1936; BUNGENBERG DE JONG AND TEUNISSEN, 1938). Since phosphatides play an important part in the protoplasmic membrane it is not very surprising that the influence of these cations on the permeability shows the same order again (BOOIJ, 1940). This hypothesis, concerning the activity of growth substances, can

also give a plausible explanation for some known properties of plant growth substances and related compounds.

1. The effect of plant growth substances is confined to certain concentrations. A stimulating effect is observed at lower concentrations, whereas higher concentrations can have an inhibiting effect. It has already been mentioned that in this case at low concentrations a turgescent effect on the protoplasmic membrane may be considered to occur which would then imply a condensing effect for the higher concentrations. Although the situation is exactly the other way about for oleate coacervates — models of the protoplasmic membrane — this effect can very occasionally be observed in lecithin-coacervates. In such a case the added substance influences the electrical charge of the coacervate.

2. In certain cases the inhibiting effect caused by an excess of growth substance can be eliminated with the aid of a little ethylene chlorohydrin. In low concentrations this compound has a condensing effect on oleate coacervates, whereas it has a turgescent effect at higher concentrations. It may be possible that ethylene chlorohydrin also has a turgescent effect on the plant cell that has been condensed by an excess of growth substance.

3. The difference between stem- and root-cells regarding their response to growth substances might be explained by the differences in nature and quantity of the sensitisers in the membrane, or by the differences in nature of the fatty acids that occur in the form of esters inside the lipid components of the membrane.

4. It is quite possible that the protoplasmic membrane of young cells has properties different from those of older tissue. This entails the possibility that the same growth substances may have a turgescent action in one case and a condensing one in the other.

5. An overdose of growth substance (see experiments on potato tubers, VELDSTRA, 1944, p. 155) not only inhibits the growth of the sprouts but also inhibits other biological processes. This may be understood if a general condensation of the protoplasmic membrane allows practically no transportation of compounds, necessary for these processes.

6. The increase in the quantity of auxin that can be extracted after treating plants with indole acetic acid (VON GUTTENBERG, 1942) can easily be explained by means of a "displacement-mechanism". It is not necessary to regard indole acetic acid as an activator of the natural auxins.

The task we now set ourselves was to see whether we could verify the hypothesis mentioned above by experiments on model systems for the protoplasmic membrane.

The question we have to face is whether the effect of plant growth substances and related compounds on oleate coacervates (models for the protoplasmic membrane — see preceding paper) will show a similar relation with their chemical structure as was found for that between structure and physiological activity. An affirmative answer would be a considerable support for the hypothesis.

The first experiments on this subject, performed by BUNGENBERG DE JONG, were encouraging. A coacervate, formed by adding a concentrated KCl solution to an oleate solution, disappeared under the influence of  $\alpha$ -naphthalene acetic acid. Quantitative experiments confirmed this observation. They were carried out as is described in the preceding paper (BOOIJ AND BUNGENBERG DE JONG, 1947). The method consists in the determination of the KCl concentration necessary to obtain a certain degree of coacervation before and after addition of the substance to be examined. The shifting is a measure for the turgescent or condensing effect (see Fig. 1).

It must be pointed out once more

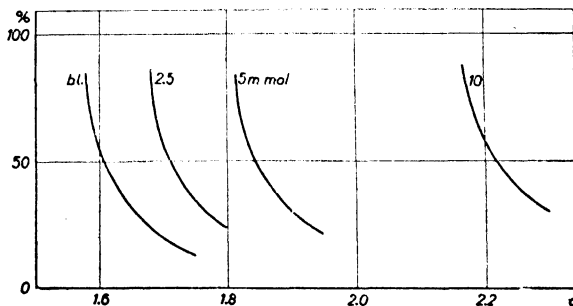


Fig. 1. Turgescent effect of naphthalene acetic acid on the coacervation of oleate by means of KCl (bl = blank, abscissa: concentration of KCl in mol/l, ordinate: volume of the coacervate layer in %).



that in our experiments the  $p_H$  is much higher than in the biological experiment. Thus in our case we study the activity of the anion, while on the other hand the biological data suggest that the non-dissociated molecule would be the active principle.

Besides growth substances plant growth inhibitors are also known, as *e.g.* compounds which check the germination of seeds (blastocholines). Especially unsaturated lactones appear to possess this property (cf. VELDSTRA AND HAVINGA, 1943, 1945). A trial attempt was made to study the effect of these blastocholines (coumarin serving as a model) on oleate coacervates. Difficulties are encountered because the medium has to be alkaline, resulting in an opening of the lactone ring. The *o*-oxy-*cis*-cinnamic acid formed has, in low concentrations at least, no influence. Therefore a lower  $p_H$  must be chosen. By using potassium acetate as a separating agent and omitting the alkali, a slight condensing effect of coumarin could be observed (Fig. 2).

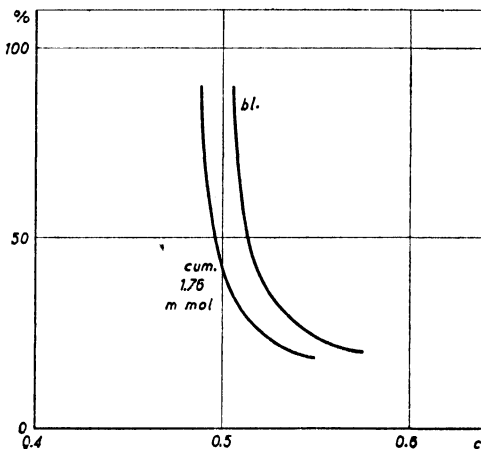


Fig. 2. Condensing effect of coumarin (abscissa: concentration of potassium acetate in mol/l).

It must be taken into account that also in this medium coumarin is slowly transformed. This transformation can readily be verified by irradiating the solution with ultraviolet light. A solution of coumarin in distilled water shows practically no fluorescence (slightly purple). On adding alkali a strong green fluorescence rapidly appears. The same happens in the presence of potassium acetate, but here the green colour is slower in appearing. The coacervate is formed while unchanged coumarin is still present. It is of course clear that this experiment only has a qualitative value. The conclusion may be drawn that coumarin has a condensing effect, but this experiment does not disclose anything about the degree of this effect. One can, however, ascertain that in

this model system the qualitative relations are as they might be expected to be according to the hypothesis.

From previous experiments on oleate coacervates it was known that many substances have a condensing effect on these coacervates. As an example of such a compound with a benzene nucleus benzylalcohol may be mentioned (BUNGENBERG DE JONG, SAUBERT, BOOIJ, 1938). This compound also proved to be active in the blastocholine test, which must mean that in a sense benzylalcohol may be

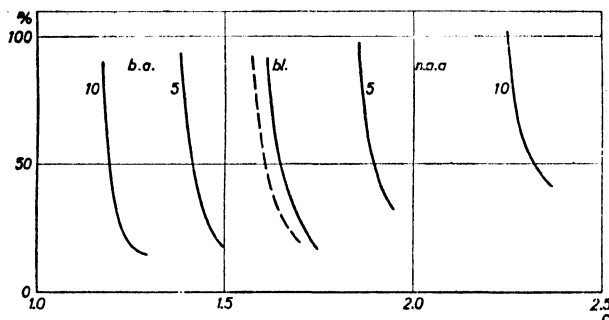


Fig. 3. Influence of benzylalcohol (b.a.) and naphthalene acetic acid (n.a.a.). A mixture of 5 m mol of benzylalcohol and 5 m mol of naphthalene acetic acid (dotted line) lies close to the blank test (bl.). Abscissa: KCl in mol/l.

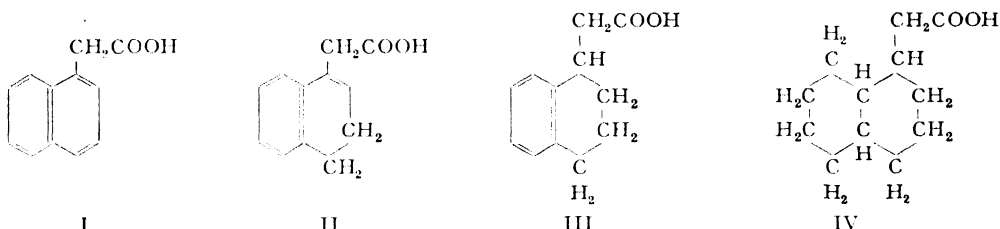
considered to be an "inhibiting substance". In this connection it is interesting to note

that the effect of benzylalcohol on oleate coacervates is clearly opposed to that of naphthalene acetic acid (Fig. 3).

After these preliminary experiments it seemed reasonable to examine whether a correlation exists between the turgescence effect of growth substances and related compounds on oleate coacervates and the physiological effect exerted on plant cells by these compounds.

## II. THE EFFECT OF GROWTH SUBSTANCES AND RELATED COMPOUNDS ON COACERVATES, IN PARTICULAR ON OLEATE COACERVATES

I. Polarographic experiments (VELDSTRA, 1944) seem to indicate that the function of the double bonds in the ring system of growth substances is not due to their reducibility (To be discussed in detail in the following paper). They rather seem to be partly responsible for the (polarographically measured) boundary-activity.



The boundary-activity decreases in the series naphthalene acetic acid, dihydro-, tetrahydro-, and decahydro-naphthalene acetic acid (I-IV) (half suppression values 9, 12, 24 and 40 micro-mol/l respectively). The physiological activity decreases parallel herewith.

The turgescence effect of these compounds on oleate coacervates, however, appears to increase in the direction already mentioned (Fig. 4).

In this case there is evidently no parallelism with the physiological effect. In a sense there is conformity with the results of the investigation on the condensing effect of non-electrolytes on oleate coacervates (BUNGENBERG DE JONG, SAUBERT, BOOIJ, 1938) in which it was demonstrated that, *i.e.*, cyclohexanol with a saturated ring system has a stronger effect than the aromatic benzylalcohol, although the latter contains an additional carbon atom.

2. The investigation on structure and activity showed that the position of the carboxyl-group in regard to the ring system is very important for the physiological activity. The ideal position is found when the

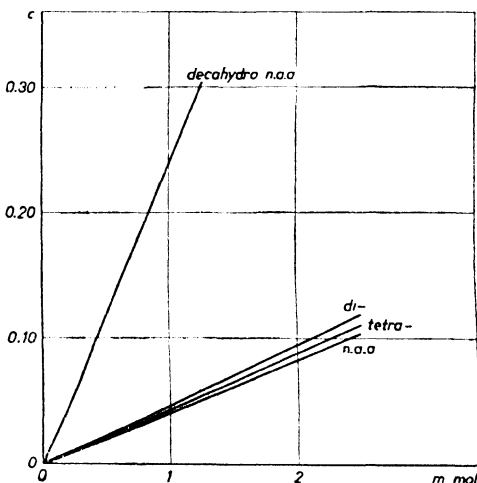
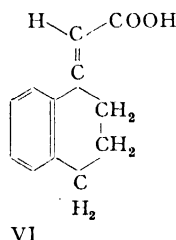
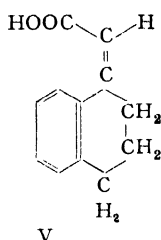


Fig. 4. Shifting of the KCl-curve in mol/l (ordinate) under influence of different hydrogenated derivatives of naphthalene acetic acid (abscissa: concentration of the added compounds).

direction of the dipole is perpendicular to the plane of the ringsystem. The difference in physiological activity between *cis*- and *trans*-cinnamic acid was explained on this base and the two forms of 1, 2, 3, 4-tetrahydronaphthylideneacetic acid (of mp 92° and 163° respectively) were considered to be an analogous pair of *cis*- and *trans*-compounds. Because of the fact that the substance of mp 92° is active in the pea-test, whereas that of mp 163° doesn't show any activity, it was deemed very probable that the former compound possesses *cis*- (V), the latter one *trans*-configuration (VI). The only difference between these acids is formed by the position of the carboxyl-group with regard to the ringsystem: this being "favourable" for the appearance of physiological activity with the *cis*-compound, "unfavourable" in this respect for the *trans*-isomer.



Experiments with the oleate coacervates show a different picture (Fig. 5). Here the higher melting isomer has a stronger effect than the lower melting one (which is slightly more active than naphthalene (I) acetic acid.

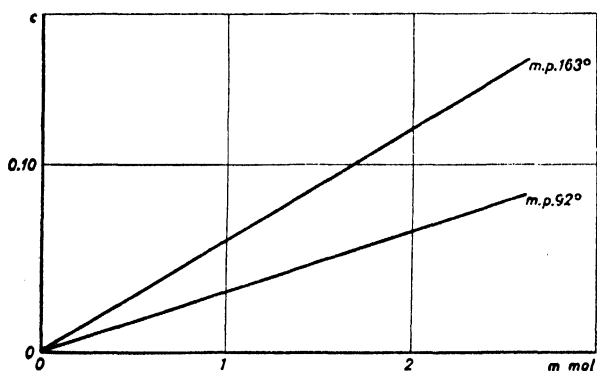


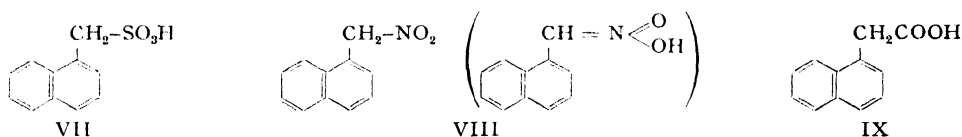
Fig. 5. Influence of the 1, 2, 3, 4-tetrahydronaphthylidene (I) acetic acids of mp 92° and 163° on the shifting of the KCl-curve (ordinate).

A comparison of the two structures for these compounds immediately shows that the length (distance between the -COOH group and the remotest part of the ringsystem) of the *trans*-compound (VI) is greater than that of the *cis*-isomer. As previous investigations revealed that a lengthening of the chain generally results in a stronger activity in the coacervate (observed for the condensing effect of organic non-electrolytes and the tur-

rescent effect of normal fatty acids) we must conclude that the compound of mp 163° — because of its higher activity in the oleate-coacervate — as compared to that of mp 92° is the "longer" one and thus possesses *trans*-configuration. The lower melting compound then must be the *cis*-isomer.

So on the one side in a quantitative sense the activities in the model system are reversed to those found with the biological object, on the other side these relations can be considered to constitute an elegant proof for the *cis*- and *trans*-structures, ascribed to the compounds of mp 92° and 163° respectively on account of their physiological activities.

3. An important question, which was examined in the earlier investigations, was whether compounds containing other groups than COOH might be active as growth substances (see VELDSTRA, 1944). For that purpose three compounds were compared, naphthalene (I) methane sulphonic acid (VII), naphthalene (I) nitromethane (VIII) and naphthalene (I) acetic acid (IX).



In the pea-test these acids proved to have an activity of 0; 4 and 100 respectively.

Such large differences are not observed in the oleate coacervates (Fig. 6) and moreover, they are exactly inversed as compared to those for the physiological effect.

4. In the homologous series of acids derived from naphthalene curious differences in activity were found in the pea-test. Naphthalene (I) acetic acid (X) proved to have a much stronger effect than  $\beta$ -naphthalene (I) propionic acid (XI) (which has a very slight activity), whereas  $\gamma$ -naphthalene (I) butyric acid (XII) again has a slight effect.

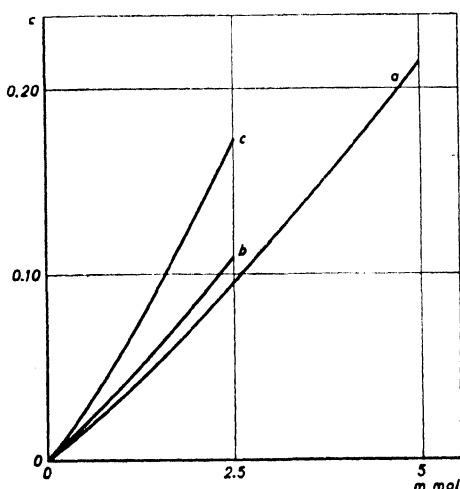


Fig. 6. Comparison of the effect of naphthalene (I) methanesulphonic acid (c), naphthalene (I) nitromethane (b) and naphthalene (I) acetic acid (a). Ordinate: shifting of the KCl-curve in mol/l.

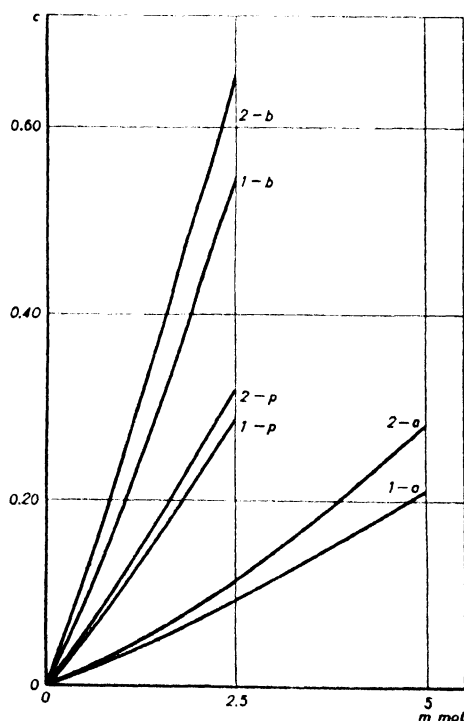
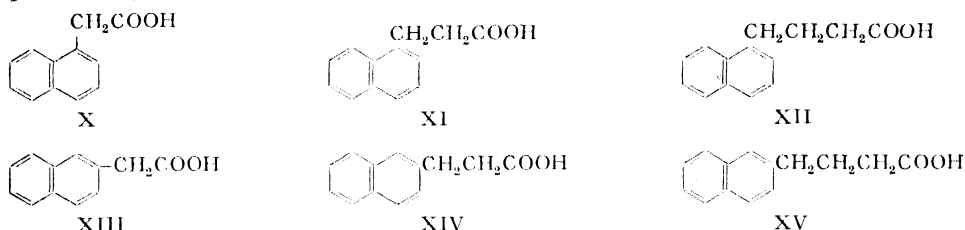


Fig. 7. Shifting of the KCl-curve (ordinate) under influence of naphthalene (I) acetic acid (1-a), naphthalene (I) propionic acid (1-p), naphthalene (I) butyric acid (1-b), naphthalene (2) acetic acid (2-a), naphthalene (2) propionic acid (2-p) and naphthalene (2) butyric acid (2-b).

So there is evidently a certain oscillation in this homologous series. Naphthalene (I) acetic acid proved to exert a slightly stronger effect than naphthalene (2) acetic acid (XIII), but, on the other hand,  $\beta$ -naphthalene (2) propionic acid (XIV) is slightly more active than  $\beta$ -naphthalene (I) propionic acid.

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The properties with regard to the oleate coacervates are much simpler (Fig. 7). Naphthalene (1) derivatives are somewhat shorter than naphthalene (2) derivatives and evidently this should be brought into relation with the decrease in activity (compare: II, 2).



Lengthening of the carbon chain of the non-polar part generally results in an increase of activity. In this way a naphthoxygroup also proved to have a stronger effect than a naphthyl-group in the corresponding position.



We compared naphthoxy (1) acetic acid (XVI) with naphthalene (1) acetic acid and naphthalene (1) propionic acid; also naphthoxy (2) acetic acid (XVII) with naphthalene (2) acetic acid and naphthyl (2) propionic acid.

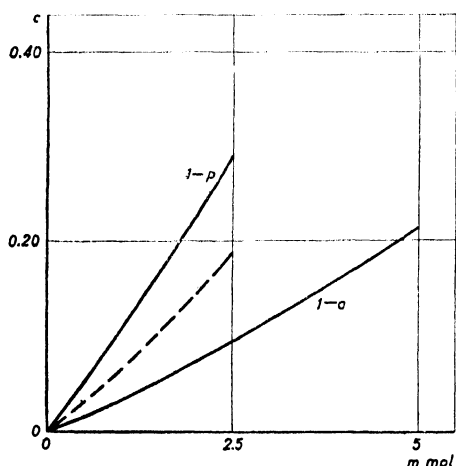


Fig. 8a. Effect of naphthoxy (1) acetic acid (dotted line) as compared with naphthalene (1) acetic acid (1-a) and naphthalene (1) propionic acid (1-p).

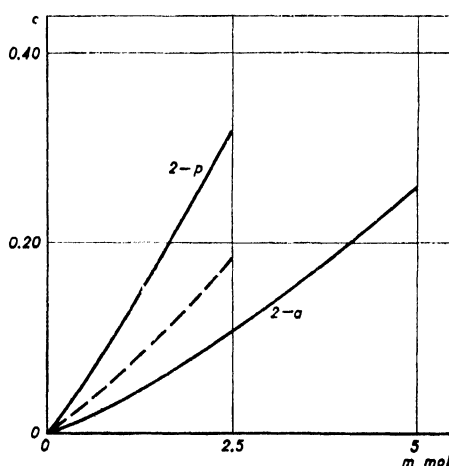
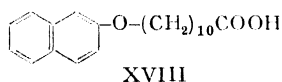


Fig. 8b. Effect of naphthoxy (2) acetic acid (dotted line) as compared with naphthalene (2) acetic acid (2-a) and naphthalene (2) propionic acid (2-p).

The result of the experiment is obvious: the length of the molecule is decisive for the degree of activity.



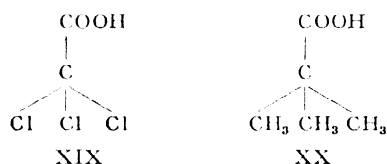
We found a remarkable result with  $\omega$ -naphthoxy (2) undecanoic acid (XVIII). Considering the great length of the molecule (about twice that of naphthoxy (2) acetic

acid) the effect is surprisingly low. We must, however, note that this acid has no biological activity. A comparison with the effect of the homologous series of fatty acid anions, however, can immediately explain the relatively low activity. Naphthoxy (2) acetic acid has a stronger effect than octanoic acid but not so strong as nonanoic acid. Therefore it may be compared with a normal fatty acid with  $8\frac{1}{2}$  C-atoms.

$\omega$ -Naphthoxy (2) undecanoic acid contains 9 C-atoms more and would therefore have to be compared with an acid of  $17\frac{1}{2}$  C-atoms. Practically speaking we would have to compare this compound with stearic acid. And our experiments indeed proved that stearic acid has a comparatively slight effect (see BOOIJ AND BUNGENBERG DE JONG, 1949, Fig. 7).

5. The ideal shape of a growth substance molecule, as deduced from earlier experiments, was supposed to be a ring system with the carboxyl-group in a peripheral position. Considerations of this kind led to experiments with trichloroacetic acid. In this acid the carboxyl-group is permanently in an "ideal" position, with regard to the lipophilic "attaching", system, consisting of the  $-C(Cl)_3$ -group. And indeed there is a very weak growth substance activity with trichloroacetic acid (in relatively high concentrations) (VELDSTRA, 1947).

It is also worth while to compare trichloroacetic acid, as regards its effect on oleate coacervates, with related compounds. The activity of acetic acid has already been mentioned in the previous paper. In low concentrations it has no effect but in very high concentrations it has a condensing influence. Trichloroacetic acid does not show any activity in low concentrations either. Therefore again — as was done with acetic acid — the method must be changed to enable measurement of the influence at high concentrations. This method has already been described in the previous



paper and it makes use of KOH instead of KCl as a means of obtaining coacervation of the oleate solutions. Then we observe (Fig. 9) that trichloroacetic acid (XIX) and trimethylacetic acid (XX) have a turgescient effect.

If a chlorine atom is introduced into each methyl-group of the latter mentioned acid the turgescient effect is decidedly stronger, as appears from the lower concentrations which now cause a comparable effect, so that the normal KCl-method can be used. This has been ascertained for  $(ClCH_2)_3-C-COOH$  and also for  $(ClCH_2)_3-C-CH_2OSO_3H$ , the activity of which approximates that of naphthalene (1) acetic acid (Fig. 10).

Although, as expected, all compounds active as growth substances show a tur-

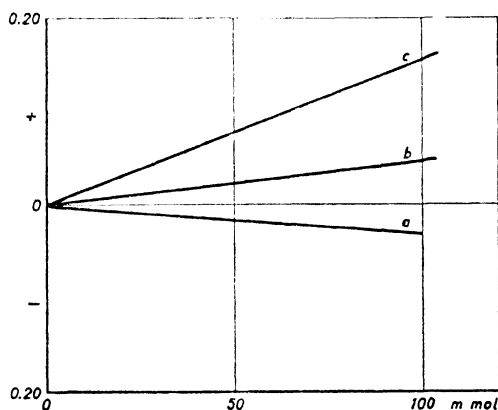


Fig. 9. Influence of acetic acid (a), trimethylacetic acid (b), and trichloroacetic acid (c) on the coacervation of oleate. Acetic acid has a condensing effect (-), the other two compounds a turgescient effect (+). Owing to the large quantities of added acids, KOH, instead of KCl, was used to induce coacervation. As usual the influence of the anions is measured in these experiments.

gescent effect on this model system of the protoplasmic membrane, little is to be seen of a more quantitative connection between the degree of this effect and the physiological activity of the compound. Only the experiment with trichloroacetic acid revealed a certain conformity.

The basis of the explanation given by BOOIJ AND BUNGENBERG DE JONG for their experiments on the influence of fatty acid anions on oleate coacervates, is formed by considerations regarding the difference and resemblance between the "substrate" and the "agent". The effect is very small if the resemblance between the acting molecule and the substrate is very great. In the extreme case — the agent is identical with the substrate, *e.g.*, the effect of the added stearate on a stearate coacervate — the effect is practically nihil. On the other hand we have to face the possibility that a total lack of conformity between "agent" and "substrate" might also produce a minimal effect. Viewed from this angle it is possible that the relations growth substance / coacervate

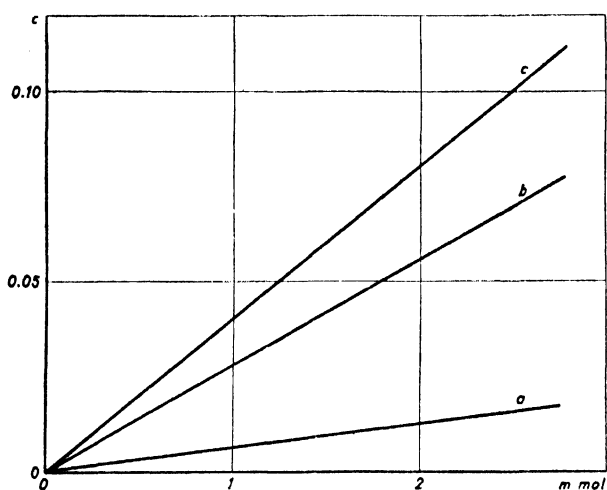


Fig. 10. Influence of (tris-chloromethyl-) acetic acid (a)\*, (tris-chloromethyl-) ethane sulphuric acid (b) and naphthalene (I) acetic acid (c) on the coacervation of oleate.

\* Its real activity very probably is higher, because it appeared that in alkaline solution this compound rather rapidly decomposes.

Furthermore, in colloid-chemical experiments the growth substances only show their turgescence effect in comparatively high concentrations, in contrast with the very low active concentration for the biological object.

In searching a better "substrate" one may be guided by these facts: the desired model is one with which the relation between the effect of naphthalene (I) acetic acid and *e.g.*, undecanoic acid is different from that in an oleate coacervate, while the former compound must preferably be active in low concentrations.

First of all we turn our attention to the stearate coacervate. In view of this model's close resemblance to the oleate coacervate it already seems probable that in this case there are few possibilities for a totally different behaviour. This surmise is confirmed by an experiment with naphthalene (I) acetic acid. It shows quite the same reactions

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and growth substance / protoplasmic membrane are not sufficiently comparable, in other words, that the oleate coacervate is not yet complete enough a model. One has to bear in mind for example, that here a protein-component of the membrane is not taken into consideration. Therefore model experiments with other model systems ("substrates") are very desirable from a theoretical point of view.

In our model experiments attention is immediately drawn to the difference between the strong effect of several normal fatty acids and the weak activity of *e.g.*, naphthalene (I) acetic acid. With the biological object this relation is exactly reversed (compare: VELDSTRA, 1947 and the following paper).

TABLE I

Shifting	of the KCl-curve (in mol/l) with the oleate coacervate under the influence of 1.25 m.mol anion of	of the temperature of separa- tion (in °C) of a desoxychol- ate coacervate under the in- fluence of 1 m.mol anion of	Shifting
0.39	decanoic acid	decanoic acid	17.5°
0.33	$\gamma$ -naphthalene (2) butyric acid		
0.305	decahydro-naphthalene ace- tic acid		
0.275	$\gamma$ -naphthalene (1) butyric acid	$\gamma$ -naphthalene (2) butyric acid	8.5
0.23	$\beta$ -naphthoyl (2) propionic acid	$\beta$ -naphthoyl (2) propionic acid	7.5
0.185	$\omega$ -naphthoxy (2) undecanoic acid		
0.175	nonanoic acid	nonanoic acid	7.0
0.16	$\beta$ -naphthalene (2) propionic acid		
0.145	$\beta$ -naphthalene (1) propionic acid	$\gamma$ -naphthalene (1) butyric acid	5.2
0.14	$\beta$ -naphthoyl (1) propionic acid	$\beta$ -naphthalene (2) propionic acid	5.0
0.115	$\alpha$ -naphthoxy (1) propionic acid	$\beta$ -naphthoyl (1) propionic acid	4.0
0.085	naphthoxy (2) acetic acid	$\beta$ -naphthalene (1) propionic acid	3.8
0.085	naphthoxy (1) acetic acid	$\alpha$ -naphthoxy (1) propionic acid	3.7
0.075	naphthalene (1) methane sul- phonic acid	naphthoxy (2) acetic acid	3.3
0.07	<i>trans</i> -tetrahydro-naphthylide- ne acetic acid	naphthoxy (1) acetic acid	3.2
0.06	<i>cis</i> -tetrahydro naphthylidene acetic acid	naphthalene (2) acetic acid	3.0
0.055	naphthalene (2) acetic acid		
0.055	octanoic acid	octanoic acid	2.7
0.055	dihydro naphthalene (1) ace- tic acid	naphthalene (1) methane sul- phonic acid	2.5
0.05	naphthalene (1) acetic acid	naphthalene (1) acetic acid	2.5
0.05	tetrahydro-naphthalene (1) acetic acid	$\beta$ -naphthalene (2) iso succinic acid	2.2
0.05	$\gamma$ -indole (3) butyric acid	<i>trans</i> -tetrahydro naphthyl- dene acetic acid	2.0
0.02	phenyl butyric acid	tetrahydro naphthalene (1) acetic acid	2.0
0.015	naphthoic acid	<i>cis</i> -tetrahydro naphthylidene acetic acid	1.8
0.01	$\beta$ -naphthalene (2) isosuccinic acid	dihydro naphthalene (1) ace- tic acid	1.8
0.01	indole (3) acetic acid	$\beta$ -naphthalene (1) iso succinic acid	1.6
0.01	naphthyl (1) sulphuric acid	phenyl butyric acid	1.4
0.005	$\beta$ -naphthalene (1) iso succinic acid		
0.005	hexanoic acid	hexanoic acid	0.8
0.005	traumatic acid	naphthoic acid	0.7
		naphthyl (1) sulphuric acid	0.7
		$\gamma$ -indole (3) butyric acid	0.1
		indole (3) acetic acid	0.0
		traumatic acid	0.0



as in oleate coacervates. Therefore the stearate coacervate does not seem a very interesting object for starting extensive experiments.

Experiments with desoxycholate coacervates promised to be of greater importance theoretically. The technique of measurement with these coacervates has already been mentioned in the preceding paper. A mixture of sodium desoxycholate (10%) and a certain solution of KOH has a well-defined temperature of separation. This point is shifted by the addition of other compounds (in case of a turgescence effect in the direction of a lower temperature) and the degree of shifting is a measure for the effect.

The influence of a large number of compounds on a desoxycholate coacervate has been examined. See Table I, in which the shifting (in °C) caused by 1 m mol (final concentration) of the added substance may be found. In this table the degree of the turgescence effect of the same compound on oleate coacervate is also given (*viz.*, the shifting of the KCl-curve in N KCl, caused by 1.25 m mol of added substance).

Of course the effects — expressed in different units — cannot be compared mutually and only the sequence of the compounds can be noted. For the greater part the sequence is similar for both models. The differences are of minor importance and, what is most important, with desoxycholate the physiologically active compounds do not occupy an exceptional position either.

The same rules as those already discussed in connection with the experiments on oleate coacervates also hold for desoxycholate. Here too the substitution of the acid group by another one is of little consequence — compare naphthalene (1) acetic acid with naphthalene (1) methanesulphonic acid — and the effect increases with a lengthening of the carbon chain — see *e.g.*, naphthalene (1) acetic acid, naphthalene (1) propionic acid and naphthalene (1) butyric acid. Therefore it cannot be said that these two “substrates” are different as far as our experiments are concerned.

Only one compound has a much stronger effect with desoxycholate than with oleate. This is  $\omega$ -naphthoxy (2) undecanoic acid. It has already been deduced that because of the length of the molecule this substance more or less resembles stearic acid. There is a great difference between desoxycholate and oleate as to the effect of the series of homologous fatty acids (BOOIJ AND BUNGENBERG DE JONG, 1949). With the oleate coacervate a minimum is observed in the neighbourhood of pentadecanoic acid, but this minimum is not found with the desoxycholate coacervate. Stearic acid has a strong turgescence effect on this model in nice agreement with the already mentioned fact that  $\omega$ -naphthoxy (2) undecanoic acid shows a great activity.

So evidently these trials with a “substrate” of a different structure were not satisfactory. Some efforts were made to “enforce” a different character on an oleate coacervate by adding certain compounds. Since most of the growth substances contain a naphthalene nucleus, we first of all tried to confer a different character upon the oleate coacervate by adding naphthalene.

Naphthalene is not easily soluble in a 2% oleate solution. The best results were obtained by adding an excess of naphthalene to the oleate solution, boiling for a short time, and putting the solution in a refrigerator. The next morning the solution is separated from crystallised naphthalene by filtration and used for the coacervation experiments.

The concentration of KCl, now required for coacervation, is considerably less than that required for an untreated oleate solution. This is due to the fact that naphthalene is a condensing substance. The influence of naphthalene (1) acetic acid and of undecanoic acid (Fig. 11) is now determined.

There is no question of a typical change in character of the coacervate. The difference between the two acids remains quite as obvious as before the treatment. In principle a condensing substance (benzylalcohol) also retains the same effect on the oleate treated with naphthalene.

Similar experiments were made on an oleate solution to which cholesterol had been added. The result of these experiments is again comparable with that obtained with untreated oleate. Neither did efforts with *m*- and *o*-cresol amount to anything. Therefore it must be concluded that it is impossible to enforce a different character on the oleate coacervate by adding condensing substances.

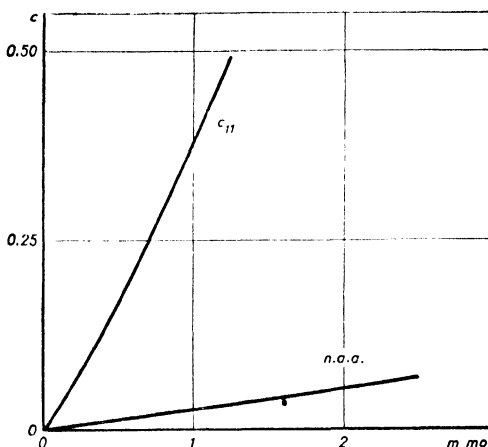


Fig. 11. Influence of naphthalene (1) acetic acid (n.a.a.) and undecanoic acid (c 11) on a coacervate condensed with naphthalene.

### III. DISCUSSION

If the results of this investigation are compared with the conclusions set down in the previous article then naphthalene (1) acetic acid appears to have a comparatively weak turgescence effect, which is about equal to the activity of octanoic acid. Of the latter acid it is known that the low activity is a result of the fact that so little is adsorbed into the soap micelles. The equilibrium concentration ( $C_E$ ) is much greater than the quantity adsorbed into the micelles ( $C_M$ )—(see BOOIJ AND BUNGENBERG DE JONG, 1947, Fig. 13).

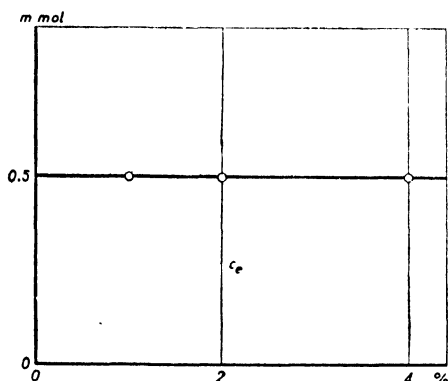


Fig. 12. The quantity of naphthalene (1) acetic acid required to cause a shifting of the KCl-curve of 0.06 mol/l in different concentrations of the oleate solution (standard 1, 2, 4%) is always the same. This means that practically no naphthalene (1) acetic acid is adsorbed into the micelles.

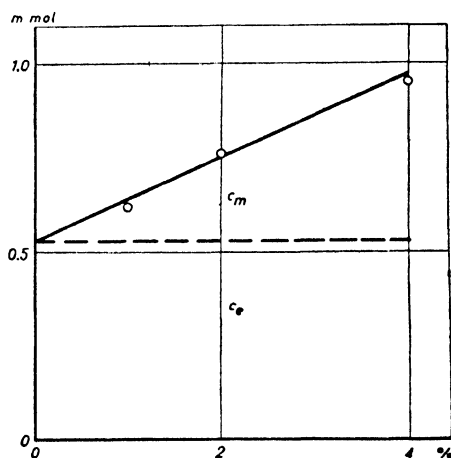


Fig. 13. For naphthalene (2) butyric acid the situation is different. Here the curve crosses the ordinate at 0.52 m mol, which must be the equilibrium concentration of this compound.

For nonanoate  $C_M$  becomes slightly larger and in the homologous series of fatty acids  $C_M$  fairly rapidly becomes practically 100%.

Of course the question arises whether the weak turgescence effect of naphthalene (1) acetic acid must also be ascribed to the low value of  $C_M$ , *i.e.*, to the small quantity of substance adsorbed into the micelles. The answer to this question can be obtained by comparing the effect for different oleate concentrations (see previous article for the method used). In Fig. 12 we find the result of this experiment: it is evident that the quantity of naphthalene (1) acetic acid adsorbed into the micelles is extremely small indeed.

Therefore we may conclude that the slight effect is due to the small quantity adsorbed.

If we regard a longer molecule, *e.g.*,  $\gamma$ -naphthalene (2) butyric acid, we see that the effect increases. An analysis of the quantity of adsorbed substance (Fig. 13) shows that here  $C_M$  is rather large. As to its effect naphthalene (2) butyric acid occupies a position between nonanoic and decanoic acid and therefore is comparable to a fatty acid containing  $9\frac{1}{2}$  C-atoms (see Fig. 7 of BOOIJ AND BUNGENBERG DE JONG, 1948). It has been calculated (see previous paper) that under certain conditions — a shifting of  $\log C_{KCl}$  by 0.04 and a concentration of the standard oleate solution of 2% — for nonanoate  $C_M$  is equal to 19% of the added quantity. For naphthalene (2) butyric acid  $C_M$  amounts to 31% under comparable conditions. As for a fatty acid of " $9\frac{1}{2}$  C-atoms" a comparable quantity would indeed be adsorbed into the micelles (see Fig. 13 of the previous paper) the important conclusion must be drawn, that for the extent of the effect the distribution of the added substance between soap micelles and medium is significant in the first place.

Efforts to enforce a different character on the oleate coacervate by adding naphthalene, cholesterol, etc. have failed. Perhaps this must be ascribed to the fact that in the soap-micelles these substances are adsorbed between the terminal methyl-groups of the parallelly arranged soap molecules. This only results in the micelles becoming thicker while the lateral distance between the soap molecules remains unchanged. HESS *et al.* (1941) made this course of events seem very probable in an investigation on the addition of benzene to a soap solution. In that case Röntgen-diagrams show that upon adsorption of benzene (see Fig. 14) the micelle only becomes thicker.

In our experiments on the addition of naphthalene, cholesterol, etc. we might also suppose that something similar happens.

It has been made probable, however, that the structure-disturbing factor acts in a direction parallel to the soap molecule. It would then be comprehensible why no influence is found upon addition of naphthalene etc.; the turgescence effect of naphthalene (1) acetic acid and of undecanoic acid remains unchanged in principle.

Another possibility is that the condensing and the turgescence effects *do* take place parallel to the soap molecules but that they are additive. In this case addition of naphthalene only results in less KCl being required for coacervation. No influence on the turgescence effect of *e.g.*, undecanoic acid will be observed. Experiments on the effects of a mixture of naphthalene acetic acid and benzylalcohol (Fig. 3) render this last conception probable.

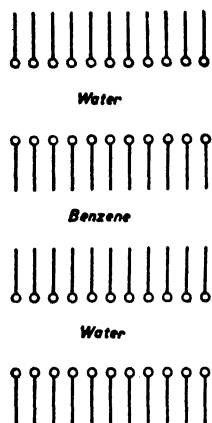


Fig. 14. Diagram of the micelles in an oleate solution to which benzene has been added (acc. to HESS, KIESSIG AND PHILIPPOFF, 1941).

In general, the result of our investigation is that for growth substances a turgescence effect on the models is observed, but that this effect is not limited to these substances and that it is therefore not specific for these physiologically active compounds. Moreover the differences in physiological activity within the group of growth substances derived from naphthalene are not reflected in the model experiments.

In the first place the relations in these experiments appear to be much simpler than in experiments on the biological activity. Quite the most important rule which may be deduced from our experiments is: the longer the non-polar part of the molecule, the stronger the activity; quite in harmony with the rules found for the turgescence effect of the fatty acids and for the condensing effect of organic non-electrolytes in previous experiments (exceptions to this rule are those molecules that closely resemble the size of the "substrate" molecules as regards their length).

Furthermore it must be noted that the growth substances must be present in a much higher concentration in the model experiments than in the biological tests in order to be efficient. Perhaps this drawback is not so very important; for as we know, the equilibrium-concentration ( $C_E$ ) is not so much what matters in these experiments but rather the amount of substance absorbed ( $C_M$ ). In this case the relation between the mass of the "substrate" and that of the agent plays an important part. In the model experiments the relative quantity of substrate is much greater than in experiments on plants.

It is, however, very important that in experiments with coacervates the normal fatty acids have a much stronger effect than the compounds possessing growth substance activity, this in contrast to the physiological experiments. A hypothesis on the mechanism of growth substance activity will in any case have to take this fact into account.

Attention must be drawn to the fact that all these experiments with coacervates took place at a high  $p_H$ . Only in that case the turgescence effect is noticeable. This means that this effect must be ascribed to the dissociated acids, i.e. to the anions. The non-dissociated molecule of all sorts of acids (see BUNGENBERG DE JONG, BOOIJ, SAUBERT, 1938) always has the same — condensing — effect. This holds for mineral and organic acids and is ascribed to the release of oleic acid from the oleate coacervate. Hence this effect is non-specific; only the concentration, but not the nature of the acid is of importance. This could also be confirmed for naphthalene (1) acetic acid. It is of course evident that the dissociation constant of the acid should not be too low, but if this condition is fulfilled the effects of all acids are completely identical.

Consequently we can conclude from our investigations on model systems that the anions are the active principles, completely different from the impression gained from biological investigations concerning growth-substance activity.

If the influence of the  $p_H$  on this activity is examined then this effect appears to be very closely connected with the dissociation-curve of the compound concerned (BONNER, 1938, cf. STRUGGER, 1932, VAN SANTEN, 1938, 1940). And the conclusion is drawn that only the non-dissociated molecule would be active, no activity being found at a  $p_H$  for which all molecules are dissociated. This would also explain why *e.g.*, naphthalene (1) methanesulphonic acid shows no growth substance activity. The dissociation constant of this substance is high, so at the  $p_H$  of the experimental conditions anions are exclusively present.

Now the problem does not become simpler. On the one hand it is improbable that the growth substances would act as co-ferments in chemical reactions (VELDSTRA, 1944;

*References p. 277.*

see also the following paper), while on the other hand it is difficult to imagine a more physico-chemical effect of the non-dissociated molecule.

Then the remark will be made that may be our model of the protoplasmic membrane (the oleate coacervate) gives a wrong impression. It is of course very well possible that the phosphatides of the protoplasmic membrane react in another manner with growth substances and fatty acids. However, it was shown that a coacervate of desoxycholate — apart from small differences — is influenced in the same way as an oleate coacervate. This is really very remarkable if the great differences between the oleate and the desoxycholate molecules are considered.

Probably the solution of this problem will be found in the fact that the driving forces which cause the absorption of the compound into the micelles are not in the first place the London-Van der Waals forces between the non-polar parts of "agent" and "substrate", but more probably the mutual forces between the water molecules, an effect which drives a non-polar compound out of the solution. With a lengthening of the carbon chain (or, in general: if the non-polar part of the molecule becomes larger) the "solubility" in water of course decreases. The relation between "agent" and "substrate" will then only be of secondary importance. It is evident that these reflections only hold for "fatty" (in the sense of: sparingly soluble in water) agents and substrates. It may be expected that for watersoluble substances other factors play a part.

For the physiological experiments this reflection has the following consequences. If the activity of a series of compounds resides on the outside of the cell, it is very improbable that we should find rules different from the simple ones known for oleate coacervates. Here *e.g.*, reference may be made to the rule of TRAUBE, which FÜHNER AND NEUBAUER (1906) find for the hemolytic influence of the normal alcohols on erythrocytes, to the influences exerted by the length of the carbon chain in hemolysis of erythrocytes with the aid of normal fatty acids (MEYER BODANSKY, 1928), and to the fact that in the germination of sweet-pea pollen under influence of organic non-electrolytes practically the same series is found again as for the effect of these compounds on oleate coacervates (BOOIJ, 1940). If the growth substances reacted only on the outside of the protoplasm then the introduction of a group with stronger acidity (as *e.g.*, in naphthalene (1) methanesulphonic acid) would scarcely have any influence on the activity (as compared with that of naphthalene acetic acid).

These facts point out that there are objections against considering the physiological effect of the growth substances to be one that exclusively influences the permeability. An important part of it most probably is localised inside the cell. The observation that non-dissociated molecules are exclusively "active" might then be explained by the fact that only uncharged molecules can easily pass the negatively charged protoplasmic membrane.

This, however, does not necessarily mean that the undissociated form of these molecules will also be the "active" one inside the cell. On the contrary, physico-chemical considerations make it far more probable that the anions will be active. Besides, a great number of the molecules will dissociate at the  $p_H$  existing in the plant cell. From this point of view the connection between the degree of dissociation of the acid active as growth substance and the degree of the activity would not be of primary character but only secondary, *viz.*, of importance for the transport of the growth substance to the place of action.

A further analysis of the growth substance activity in connection with the results

of a comparative investigation on models and biological objects will be given in the next publication.

### SUMMARY

1. In order to test the hypothesis that the action of synthetic compounds active as plant growth substances is localized largely in the protoplasmic membrane (influencing permeability), the influence of this type of compounds upon coacervates was studied.

In case of the hypothesis being valid, a turgescence action upon the coacervate would be expected, running parallel with the physiological activity.

2. *a.* In the series of naphthalene (1) acetic acid, di-, tetra- and decahydronaphthalene (1) acetic acid there is an increase of turgescence action upon oleate coacervates.

In botanical objects, however, physiological activity decreases in the same sequence.

*b.* The turgescence action upon an oleate coacervate in *trans*-, 1, 2, 3, 4-tetrahydronaphthylidene (1) acetic acid is stronger than in its *cis*-isomer, whereas, in contrast with the *cis*-form, the *trans*-isomer exerts no growth promoting action.

*c.* Replacement of the carboxyl-group by a group of stronger acidic character causes a decrease of growth substance activity; with the oleate coacervate the reverse is observed.

*d.* In the homologous series of naphthalene (1)-acetic acid, -propionic acid and -butyric acid the influence upon the oleate coacervate increases (due to the fact that — in this sequence — the added substance is increasingly adsorbed by the soap micelles). The physiological activity in these series shows a decline, accompanied by a certain oscillation.

*e.* With the oleate coacervate the relative activities of acetic acid and trichloroacetic acid are in accordance with the expectations based on the hypothesis.

3. In general the action of the anions of acids with growth substance activity and related compounds on the oleate coacervate can be explained by the rules given previously (BUNGENBERG DE JONG).

4. All the compounds with growth substance activity show the expected turgescence effect in coacervate systems (serving as a model for the protoplasmic membrane); in a quantitative measure, however, the course is not parallel to the physiological activity, but just the reverse.

5. This difference will have to be explained either by the fact that the relations growth substance/coacervate and growth substance/protoplasmic membrane are not sufficiently comparable (in other words, that the oleate coacervate as a model lacks completeness) or by the circumstance that an essential part of the growth substance activity — in contrast with the hypothesis — is not localized in the membrane.

On the strength of several arguments given the latter explanation must be considered to be the likeliest one.

6. It seems probable that the growth substance action unfolds itself largely in the protoplasm and that therefore the growth substances will have to pass the membrane.

In that case the influence of the pH on growth substance activity becomes plausible, as the non-dissociated molecules of the acids permeate much more easily than their anions.

7. A more detailed analysis of growth substance action on the basis of results of comparative investigations on models and biological objects is presented in the next paper.

### RÉSUMÉ

1. Pour vérifier l'hypothèse que l'action des substances synthétiques agissant en tant qu'hormones végétales de croissance, est localisée essentiellement dans la membrane protoplasmique (modifiant ainsi la perméabilité), l'influence de ces substances sur des coacervats a été étudiée. Au cas où l'hypothèse serait juste, on devrait s'attendre à une action turgescence vis à vis du coacervat, et qui serait parallèle à l'activité physiologique.

2. *a.* Dans la série de l'acide 1-naphthalène-acétique, et des acides di-, tétra- et déca-hydronaphthalène-1-acétique, on trouve un accroissement de l'action turgescence sur les coacervats d'oléate. Toutefois, l'activité physiologique vis à vis de matériel végétal décroît dans le même ordre.

*b.* L'action turgescence sur le coacervat d'oléate de l'acide *trans*-, 1, 2, 3, 4-tétrahydronaphthylidène-1-acétique est plus forte que celle de son isomère *cis*, alors que, en contraste avec la forme *cis*, l'isomère *trans* n'exerce aucune action de croissance.

*c.* Le remplacement du groupement carboxyle par un groupement à caractère plus acide, provoque une diminution de l'activité comme substance de croissance; c'est l'inverse qui se produit dans l'action sur un coacervat d'oléate.

*d.* Dans la série homologue des acides naphthalène-1-acétique, naphthalène-1-propionique et naphthalène-1-butyrique, l'influence sur un coacervat d'oléate croît (ce qui est dû au fait que, dans

*References p. 277.*

cet ordre, les substances sont de plus en plus adsorbées par les micelles de savon). L'activité physiologique dans cette série diminue, accompagnée d'une certaine oscillation.

e. Les activités relatives de l'acide acétique et de l'acide trichloroacétique sur le coacervat d'oléate sont en accord avec l'hypothèse initiale.

3. En général, l'action, sur le coacervat d'oléate, des anions des acides doués d'une activité de substance de croissance, peut être expliquée par les règles données précédemment (BUNGENBERG DE JONG).

4. Tous les composés doués d'une activité de substance de croissance manifestent un effet de turgescence vis à vis des coacervats (lesquels constituent un modèle de la membrane protoplasmique); quantitativement néanmoins, cet effet n'est pas parallèle à l'activité physiologique, mais lui est inverse.

5. Cette différence devrait être expliquée soit par le fait que les relations entre les substances de croissance et le coacervat d'une part, et les substances de croissance et la membrane protoplasmique d'autre part, ne sont pas suffisamment comparables, (en d'autres termes, que le coacervat d'oléate n'est pas un modèle suffisant) ou par le fait qu'une partie essentielle de l'activité de la substance de croissance, contrairement à l'hypothèse émise, n'est pas localisée dans la membrane. C'est cette dernière explication qui semble la plus plausible.

6. Il semble probable que l'action de la substance de croissance s'exerce surtout dans le protoplasma et que, par conséquent, la substance de croissance doit franchir la membrane. Dans ce cas, l'influence du  $pH$  sur l'activité de la substance de croissance devient compréhensible, car les molécules non dissociées des acides traversent la membrane beaucoup plus facilement que leurs anions.

7. Une analyse plus détaillée de l'action des substances de croissance, analyse basée sur les résultats d'études comparatives sur des modèles et sur du matériel biologique, est donnée dans le mémoire suivant.

#### ZUSAMMENFASSUNG

1. Um die Hypothese, dass die Wirkung synthetischer Verbindungen, die als Pflanzenwachstumsstoffe aktiv sind, hauptsächlich in der Protoplasamembran lokalisiert ist (also die Permeabilität beeinflusst), zu prüfen, wurde der Einfluss dieser Art von Verbindungen auf Koazervate untersucht.

Im Falle der Gültigkeit der Hypothese müsste eine Turgeszenzwirkung auf die Koazervate auftreten, die erwartungsgemäss parallel mit der physiologischen Aktivität verlaufen sollte.

2. a. In der Reihe Naphtalin(1)essigsäure, Di-, Tetra- und Dekahydronaphtalin(1)essigsäure tritt eine Zunahme der Turgeszenzwirkung auf Oleatkoazervate auf.

Bei botanischen Objekten nimmt die physiologische Aktivität jedoch in derselben Reihenfolge ab.

b. Die Turgeszenzwirkung auf Oleatkoazervat von *trans*-, 1, 2, 3, 4, tetrahydronaphtylyden(1)essigsäuren ist stärker als die des *cis*-Isomeren, während — im Gegensatz zur *cis*-Form — das *trans*-Isomere keine wachstumsfördernde Wirkung hat.

c. Ersetzen der Carboxylgruppe durch eine Gruppe mit stärker saurem Charakter verursacht eine Abnahme der Wuchsstoffwirkung; beim Oleatkoazervat wird die entgegengesetzte Wirkung beobachtet.

d. In der homologen Reihe Naphtalin(1)essigsäure, -propionsäure, und -buttersäure nimmt der Einfluss auf das Oleatkoazervat zu (diese Zunahme beruht darauf, dass die zugefügte Substanz in dieser Reihenfolge stärker von den Seifenmicellen adsorbiert wird). Die physiologische Aktivität in dieser Reihe zeigt eine Abnahme, die von einer gewissen Oszillation begleitet ist.

e. Beim Oleatkoazervat sind die relativen Aktivitäten von Essigsäure und Trichloressigsäure in Übereinstimmung mit den Erwartungen auf Grund der Hypothese.

3. Im allgemeinen kann die Wirkung von Anionen der Säuren mit Wuchsstoffaktivität und von verwandten Verbindungen auf das Oleatkoazervat mit den Regeln, die bereits früher angegeben wurden (BUNGENBERG DE JONG), erklärt werden.

4. Alle Verbindungen mit Wuchsstoffaktivität zeigen den erwarteten Turgeszenzeffekt bei Koazervatsystemen (die als Modell für die Protoplasamembran dienen); quantitativ betrachtet verläuft die Aktivität jedoch nicht parallel mit der physiologischen Aktivität, sondern gerade umgekehrt.

5. Dieser Unterschied muss entweder dadurch erklärt werden, dass die Beziehung Wuchsstoff/Koazervat und die Beziehung Wuchsstoff/Protoplasamembran nicht genügend vergleichbar sind (mit anderen Worten, dass das Oleatkoazervat als Modell nicht vollständig ist), oder dadurch, dass ein bedeutender Teil der Wuchsstoffaktivität — im Gegensatz zur Hypothese — nicht in der Membran lokalisiert ist.

Auf Grund der Beweiskraft verschiedener angegebener Argumente muss die letztere Erklärung als die wahrscheinlichere betrachtet werden.

6. Es scheint wahrscheinlich zu sein, dass die Wuchsstoffaktivität sich hauptsächlich im Protoplasma entfaltet, und dass deshalb der Wuchsstoff erst die Membran passieren muss.

References p. 277.

In diesem Falle wird der Einfluss des pH auf die Wuchsstoffaktivität plausibel, da die undissoziierten Säuremoleküle sehr viel schneller diffundieren als ihre Anionen.

7. Eine ausführlichere Analyse der Wuchsstoffaktivität auf der Grundlage der Ergebnisse vergleichender Untersuchungen an Modellen und biologischen Objekten wird in der folgenden Arbeit gegeben.

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## RESEARCHES ON PLANT GROWTH REGULATORS

XVII. STRUCTURE AND ACTIVITY.  
ON THE MECHANISM OF THE ACTION III

by

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## I. INTRODUCTION

Preceding investigations concerning structure and activity of plant growth substances and their mutual relation led to the conception that, as to the structure, a peculiar relation (also to be taken in a spatial sense) between lipophilic basal ring system and hydrophilic acid group is essential for a high growth activity (VELDSTRA, 1944). In this respect the required spatial structure reminds strongly of the relations in the group of wetting agents and penetrants (cf. page 280). Considering the actions to be expected from such types of compounds, a physico-chemical mechanism of action was deemed the more probable one, and, based on the colloid-chemical investigations of BUNGENBERG DE JONG *et al.*, the working hypothesis was developed that this action would consist of an influencing of the permeability (intrability) of the protoplasmic membranes.

As to the relation structure/activity these views were further put to the test with more extensive material, which came at our disposal from own investigations and from the literature, whereas with respect to the mechanism of the action ample investigations were carried out with the aid of model-systems (HAVINGA, VELDSTRA, 1948; BOOIJ, BUNGENBERG DE JONG, 1949; BOOIJ, VELDSTRA, 1949).

After a discussion of the results of the structural investigations, the consistencies of the latter investigations on model systems for the view concerning the action will be considered, also in connection with studies on biological systems and opinions developed elsewhere.

## II. STRUCTURE AND ACTIVITY

The essential structural requirements for compounds to possess a high growth activity were given in the former investigations by

A. Basal ring system (non-polar part) with high interface activity.

B. Carboxyl group (polar part)—in general a group of acidic character—in such a spatial position with respect to the ring system, that on adsorption of the active molecule to a boundary (the non-polar part playing the most important rôle) this functional group will be situated as peripherically as possible.

*References p. 311/312.*

It was now considered to what extent lipophilic structures, other than those studied up till now (benzene-, naphthalene- or indolenuclei, or closely related structures) might be capable of filling the function of "attaching system" (requirement A), simultaneously carrying the COOH group in a position required according to B.

The simplest type conceivable which would meet these requirements as far as B is concerned, is without doubt trichloroacetic acid (I), though it very probably will be very much too water-soluble.

Here the "special" form of the requirements for a growth substance, as formulated by KOEPFEL, THIMANN, AND WENT (1938) is completely abandoned and attention is paid only to both demands to be made generally as expressed under A and B.

As already briefly communicated elsewhere (VELDSTRA, 1947) trichloroacetic acid proved to possess a very low activity in the pea test (active at  $5 \cdot 10^{-3}$  mol/l)\*. Though there is thus an indication of some activity, on a closer examination this evidence is not wholly conclusive because of the high concentration required. Therefore it is not absolutely sure that the effect is based

on the same mechanism as that of the normal growth substances. Nevertheless the effect of this simple "ideal" type of acid in itself remains interesting, also in connection with other properties shown by it, as e.g., its action on proteins, and with the behaviour of "spatial related" branched fatty acids, to be discussed hereafter (see page 280).

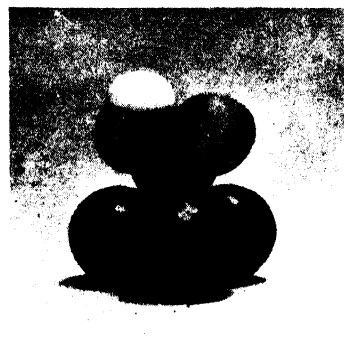
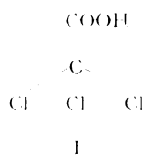


Fig. 1. STUART model of trichloroacetic acid

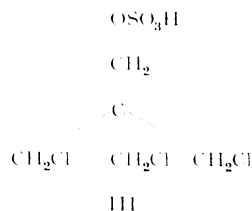
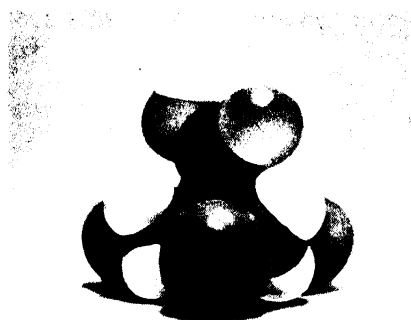
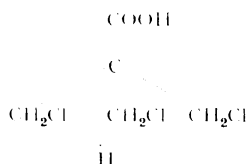


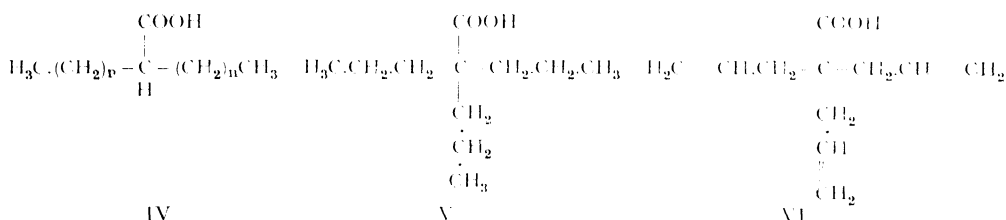
Fig. 2. STUART model of tris-(chloromethyl)-acetic acid

In order to approximate more closely the molecular dimensions of the highly active compounds, such as naphthalene acetic acid, and to obtain a higher attaching power, we investigated tris-chloromethyl acetic acid (II) and the related tris-chloromethyl ethane sulphuric acid (III).

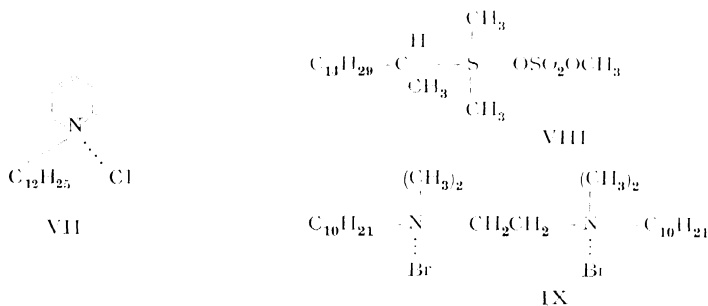
\* The acids investigated in the pea test were always used in the form of their potassium salts.  
References p. 311/312.

No activity in the pea test could be detected, however. As to the most interesting compound of the pair, namely tris-chloromethyl acetic acid, no definite conclusion could be arrived at because it turned out that a solution of its potassium salt rather rapidly decomposed with loss of the carboxyl group. So it proved to be impossible to determine exactly whether this compound would be active or not. The half suppression value determined polarographically ( $\approx > 240$ ) indicated however that very probably also with this compound the surface activity has not yet become sufficiently high.

As the effect of naphthalene acetic acid in the oleate coacervate is comparable with that of octanoic acid (cf. preceding paper) we reinvestigated the latter acid in the pea test over a wide range of concentrations. With the higher concentrations ( $\approx 5 \cdot 10^{-3}$  mol/l) the effect described before (VELDSTRA, 1947) was observed again, but in lower concentrations not the slightest inward curvatures were obtained. Further we investigated related fatty acids with branched chains, whose structures resemble more closely that of trichloroacetic acid and thus meet better the requirement B (page 278) than in the case of normal fatty acids. In fact in this way we are dealing with prototypes of the stronger wetting agents (cf. YOUNG, COONS, 1945; PRICE, 1946).



Of these acids: di-n-propylacetic acid (IV,  $n = 2$ ), di-n-butylacetic acid (IV,  $n = 3$ ), di-n-amylacetic acid (IV,  $n = 4$ ), tri-n-propylacetic acid (V) and triallylacetic acid (VI), none did show any distinct physiological activity however. Only in the high concentrations ( $10^{-2} - 10^{-3}$  mol/l), near the toxic level, there appeared weak curvatures as described for comparable concentrations of the normal  $\text{C}_8 - \text{C}_{14}$  fatty acids (VELDSTRA, 1947), the objects in the pea test becoming more or less transparent. In this latter respect the branched fatty acids act more rapidly than their normal isomers. These observations induced us to study the effect of some—both mutually as with respect to the fatty acids—structurally quite unrelated wetting agents, such as the compounds VII-IX.



These compounds proved to be very toxic (already with  $4 \cdot 10^{-5}$  mol/l), but quite near to these concentrations the transparency of the objects in the pea test, as mentioned before, was observed, accompanied by weak curvatures of the type obtained with the

fatty acids in high concentrations. Particularly with compound IX, where the hydrophilic part is located in the centre of the lipophilic chain (cf. page 297) these effects were observed (see Fig. 3). In this way it becomes more probable that these curvatures do not represent a normal physiological effect, but are rather correlated with an—in this case—exaggerated form of a factor, also playing a part normally, namely with an abnormal uptake of water.

Thus with these “biped” and “tripod” compounds no significant activity could be observed and up till now this remains wholly restricted to the types derived from naphthalene, indole, substituted benzene or related ring systems.

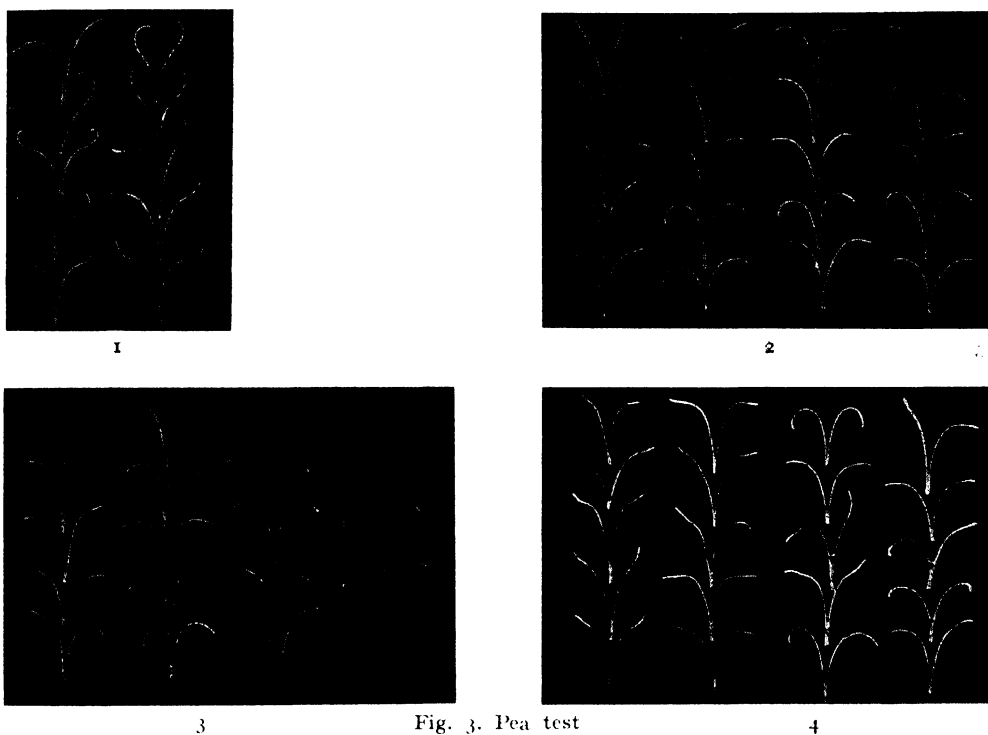
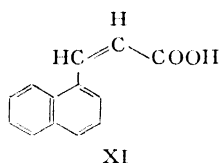
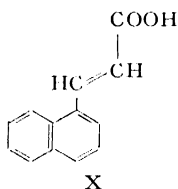


Fig. 3. Pea test  
 1. Naphthalene acid acetic, from 1. to r.: 4;  $1 \cdot 10^{-5}$  mol/l  
 2. Compound VII  
 3. Compound VIII  
 4. Compound IX  
 } 4; 2; 1;  $0.4 \cdot 10^{-5}$  mol/l

The bearing of these findings on the discussion of the mode of action of the growth substance will be dealt with afterwards (page 297).

As to the spatial relation of ring system and carboxyl group in the side chain some more interesting facts could be added to those already known (*cis*- and *trans*-cinnamic acid, tetrahydronaphthylidene acetic acids). As to the tetrahydro-naphthylidene acetic acids, it was already communicated in the preceding paper that their interactions with the oleate coacervate constituted a proof for the *cis*- and *trans*-structures ascribed before to the compounds of m.p.  $92^\circ$  and  $163^\circ$ , respectively, on account of the different behaviour in the pea test (cf. VELDSTRA, 1944). Moreover a very conclusive proof now

has been given by HAVINGA AND NIVARD (1948) by means of the ultraviolet absorption spectra and comparison of the difference found with that observed for comparable pairs of *cis*- and *trans*-acids. To investigate an analogy of the couple *cis*- and *trans*-cinnamic acid, *trans*  $\beta$ -naphthalene(1)acrylic acid (X) was synthesized and found to be totally inactive in the pea test.



By irradiation with ultraviolet light an isomer could be obtained (m.p. 141°, HAVINGA, NIVARD, 1948), which could be regarded as the *cis*-form (XI), and which, like

*cis*-cinnamic acid, was indeed distinctly active (Fig. 4). The typical relation between spatial structure and activity as derived from the former investigations here once more comes to the fore. It is interesting that quite recently SEXTON AND TEMPLEMAN (1948), in a study of the differential effects of 2-benzoyl-benzoic acid and its derivatives, found that also with these compounds similar relations may be of importance.

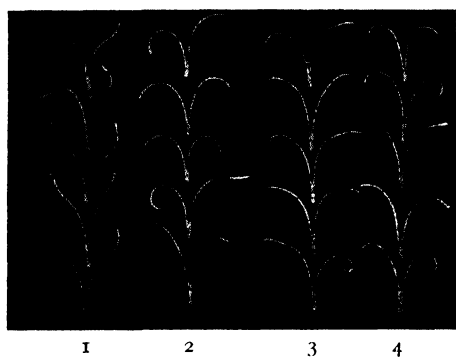
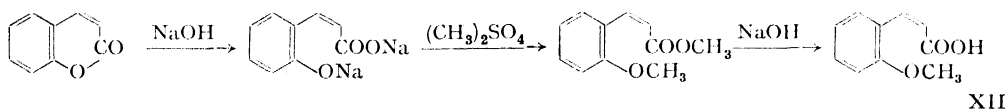


Fig. 4. Pea test

1; 3: *cis*-Naphthalene(1)acrylic acid 10 and  $4 \cdot 10^{-6}$  mol/l  
2; 4: *trans*-Naphthalene(1)acrylic acid 10 and  $4 \cdot 10^{-6}$  mol/l

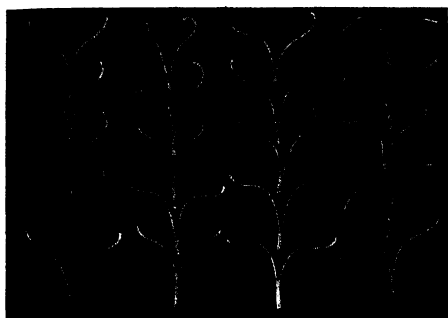
HAAGEN-SMIT AND WENT (1935) had already investigated o-methoxy *cis*-cinnamic acid (XII) and found it to be slightly active in the pea test. As this compound was highly interesting to us because of its relation to the growth inhibiting coumarin and the bearing thereof on the general problem of the struc-

tural relation between growth stimulating and inhibiting compounds (cf. VELDSTRA, HAVINGA, 1943, 1945) we prepared this cinnamic acid derivative by means of the following reactions (STOERMER, FRIEMEL, 1911):

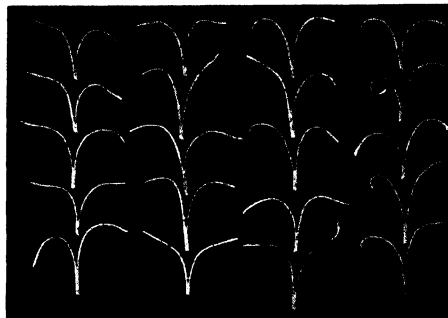


By assaying this compound in the pea test the results of HAAGEN-SMIT AND WENT could be confirmed (Fig. 5) and thus it proves possible by means of a simple series of reactions to convert a growth inhibiting substance into a stimulating one. Of course it would have been still more interesting to investigate the parent acid of the lactone coumarin, namely o-hydroxy-*cis* cinnamic acid. But as already described previously (VELDSTRA, HAVINGA, 1943) this failed, as in a solution of this acid coumarin is formed immediately.

In a comparable way *cis*-2-methoxy- $\beta$ -naphthalene(1) acrylic acid (XIV) was obtained from 5.6-benzocoumarin (XIII). For the latter compound it had already been



1

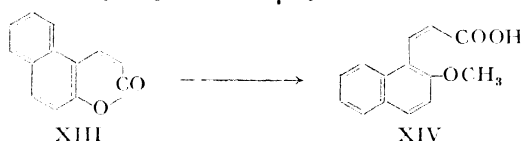


2

Fig. 5. Pea test

1: *cis*-o-methoxy-cinnamic acid from 1. to r.2: *cis*-2-methoxy-naphthalene(1)acrylic acid from 1. to r.}  $50; 25; 10; 4 \cdot 10^{-5}$  mol/l

established that its inhibiting activity was weaker than that of coumarin (VELDSTRA, HAVINGA, 1943). The methoxy-naphthalene acrylic acid, derived thereof

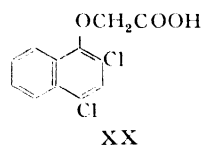
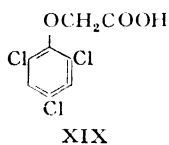
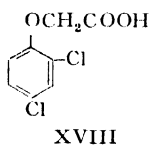
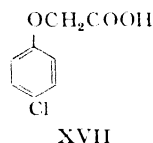
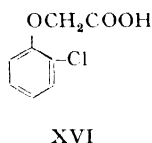
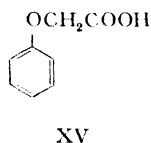


was found to be active, but to a much lesser extent than the methoxycinnamic acid (cf. Fig. 5).

Maximal activity both in a stimulating and in an inhibiting sense thus seems to be connected with a rather well-defined molecular size, as *e.g.*, also appears from the fact that, compared with coumarin, the chloro-coumarins generally are weaker inhibitors (cf. AUDUS, QUASTEL, 1947; VELDSTRA, NAUTA, 1949).

As the growth substances have the same hydrophilic part in common, namely the COOH group, the molecular size required for maximal activity implies a definite lipophilic part in balance with the hydrophilic carboxyl group\*.

One arrives at the same conclusion when comparing phenoxy-acetic acid derivatives mutually or with conformable derivatives of naphthoxy-acetic acid.



Starting with phenoxy-acetic acid (XV), itself but very weakly active in the pea

\* Henceforth this balance between hydrophilic and lipophilic (hydrophobic) parts of similar polar/non-polar compounds (amphipatic compounds, cf. HARTLEY, 1941) will also be denoted as HL-balance.

test, introduction of chlorine atoms in the o- (XVI), p- (XVII) or in the o- and p- (XVIII) positions simultaneously causes in the same order an increase in activity, so that 2,4-dichlorophenoxy-acetic acid is quite as active as naphthalene acetic acid (cf. Fig. 6).

If their interface activity is measured polarographically, it proves to increase in the same sense, the activity of 2,4-D in this respect also being wholly comparable with that of naphthalene acetic acid (half suppression values (HSV): phenoxy-acetic acid 130; o.chlorophenoxy-acetic acid 17; p.chlorophenoxy-acetic acid 14; 2,4-dichlorophenoxy-acetic acid 7; naphthalene-acetic acid 5).

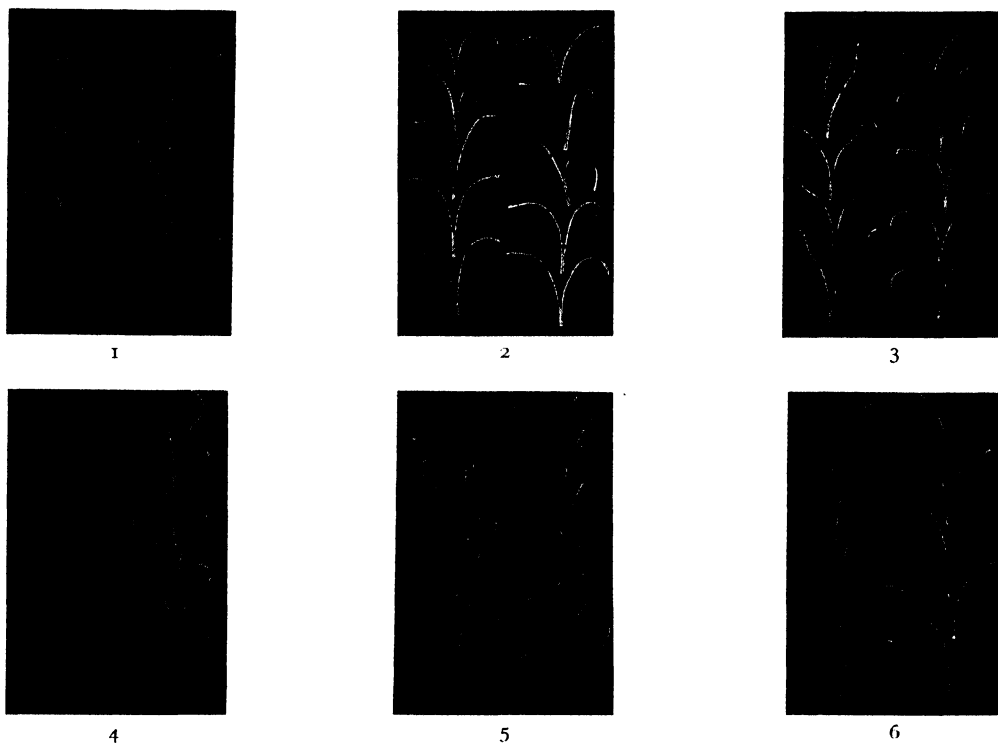


Fig. 6. Pea test

- |                                       |   |
|---------------------------------------|---|
| 1. naphthalene-acetic acid            | from 1. to r.: 4; $1 \cdot 10^{-5}$ mol/l<br>from 1. to r.: 10; $4 \cdot 10^{-5}$ mol/l |
| 2. phenoxy-acetic acid                |   |
| 3. o.chlorophenoxy-acetic acid        |   |
| 4. p.chlorophenoxy-acetic acid        |   |
| 5. 2,4-dichlorophenoxy-acetic acid    |   |
| 6. 2,4,6-trichlorophenoxy-acetic acid |   |

Apparently in this series the above-mentioned balance between lipophilic (non-polar) and hydrophilic (polar) part, characteristic for maximal activity, is approximated in the best way with 2,4-D. For if one more chlorine atom is introduced, the resulting 2,4,6-trichlorophenoxy-acetic acid (XIX) proves to be practically inactive in the pea test, though its interface activity still has increased (HSV: 4,8). We believe that in the same sense in 2,4-dichloronaphthoxy-acetic acid (XX) the balance has turned too much towards the lipophilic side, this compound also possessing a very low physiological activity.

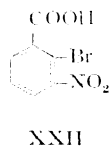
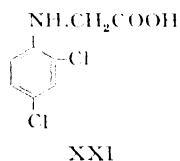
We consider it probable that with this HL-balance the real meaning of the "high interface activity of the non-polar part" (requirement A., page 287) is detailed more strictly, as this interface activity now too is brought in relation with the carboxyl group and a certain limit can be indicated. The interface activity namely not only can be too low (from which side in our former investigations this problem was mainly viewed), but also too high.

In this way the requirements for maximal growth substance activity are considered to consist of a double relation between non-polar and polar parts of the active molecule, a physico-chemical and a spatial one.

A more extensive discussion of this relation will follow after comparison of the action of growth substances and related compounds on the oleate coacervate and on biological systems (cf. page 290).

After the appearance of our first paper on the relation between chemical structure and growth substance activity, in which the active compounds known up till then were summarized, a vast amount of compounds has been investigated as to their growth activity in different centres—but mainly in the U.S.A.—(ZIMMERMAN *et al.*, 1941–1943; 1944; THOMPSON *et al.*, 1946; TEMPLEMAN, SEXTON, 1946; NEWMAN *et al.*, 1947; cf. TINCKER, 1940, 1948).

For the study of the relation structure/activity it is rather a pity that the tests used, for reasons discussed before (VELDSTRA, 1944, page 100) do not permit to draw conclusions in this respect. For this purpose the most interesting series will also have to be investigated in the pea test. Apart from the phenoxyacetic acid derivatives already mentioned, only a few of them were investigated by us up till now. For the moment we will only state that the activity of N-2,4-dichlorophenylglycine (XXI) is comparable with that of 2,4-D and naphthalene-acetic acid. Thus apparently also this type of side chain meets the requirements.



Very interesting both in a theoretical sense and to practical applications are the effects shown by substituted benzoic acids. (ZIMMERMAN, HITCHCOCK, 1942). So 2-bromo-3-nitro-benzoic acid (XXII) was reported to be mildly active for cell-elongation. We reinvestigated this compound in the pea test and found it to be active, though but very weakly.

This means that for the first time activity (let it be a very small one) is here encountered in a compound which does not fit the rule of KOEFLI, THIMANN, AND WENT, that for the display of growth activity at least one carbon atom is required between ring system and carboxyl group or at first sight does not show the spatial relation between non-polar and polar part as we described. These requirements for *maximal activity* thus do not seem to be a "conditio sine qua non" for the appearance of growth activity in general. From a theoretical point of view this is a very interesting observation and therefore these questions were studied more in detail, also with compounds not investi-





Fig. 7. Pea test

1. naphthalene acetic acid, from l. to r.: 4; 1; 0.4;  $0.1 \cdot 10^{-5}$  mol/l  
 2. 2-bromo-3-nitro-benzoic acid from l. to r.: 50; 40; 30;  $20 \cdot 10^{-5}$  mol/l

gated in this respect up till now. The results of these investigations will be discussed in a following paper.

### III. ON THE MECHANISM OF THE ACTION

The investigations concerning the effect of growth substances on model systems, particularly on the oleate coacervate (BOOIJ, VELDSTRA, 1949), showed that the type of action answered expectations, but that the quantitative relations between opening effect and physiological activity in the series of naphthalene and indole derivatives were just the reverse of those one would expect on account of the view that the growth substances mainly act on the protoplasmic membranes. These divergences might be caused by the fact that the model system used does not correspond completely enough with the system physiologically reacting. On account of some arguments given in the preceding paper (BOOIJ, VELDSTRA, 1949, page 274) it was considered more probable, however, that with the view of growth substances influencing intrability/permeability the essential part of their activity had not yet been indicated and that most probably this had to be looked for within the protoplasm itself.

In order to shorten the discussion on these two possibilities and to show whether it was justified or not to base conclusions on the effects observed with the oleate coacervate, it was deemed highly important to compare the effect of some series of compounds on the coacervate with those on a biological system of such a type that one could almost certainly ascribe the effect to an interaction with protoplasmic boundaries.

One case of conformity between the effects obtained with the oleate coacervate and a biological system has already been demonstrated. The first part of the typical curve describing the opening action of normal fatty acids on the coacervate is namely reflected in the curvatures shown by these acids in the pea test (VELDSTRA, 1947). The relative figure is reproduced here once more (Fig. 8).

This parallelism, though already indicating that some biological system reacts comparably with the coacervate, does not yet furnish, however, such decisive arguments for the present discussion that it allows a choice in the above-mentioned sense. As outlined on page 280, it must moreover be taken into consideration that with these effects of the fatty acids in the pea test still other factors than those of primary importance for a normal physiological reaction may play a rôle. For this reason the relations

found are more significant for a discussion on the *type* of action than for one on its *localization*.

For the latter purpose it seemed to us that the border tissue of the red beet (*Beta vulgaris rubra*) would offer an attractive possibility. This object has already been used previously to study the action of compounds on the plasmic boundaries. If such an action takes place, causing disturbance of the semi-permeability of endo- and ectoplasmic membranes, the colouring matter—contained in the vacuole—can leave the

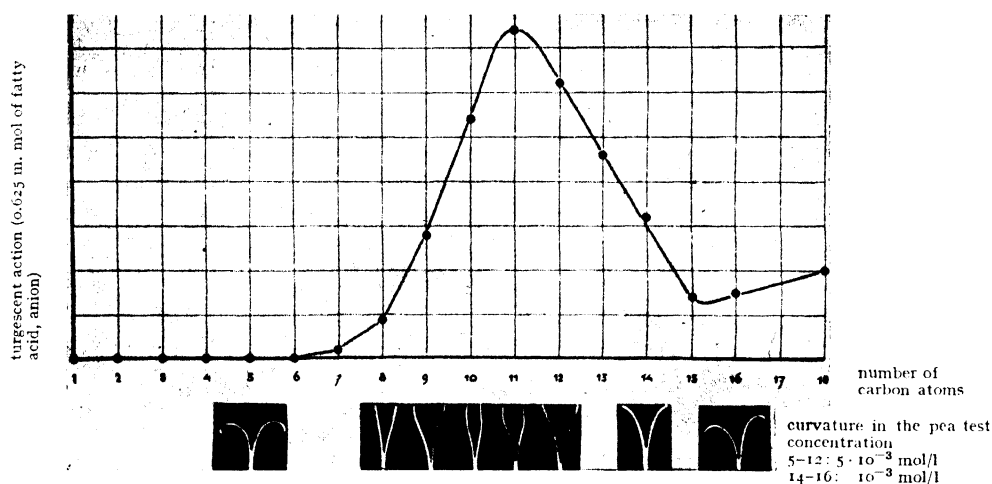


Fig. 8. Turgescence (opening) action on the oleate coacervate and growth activity in the pea test of normal fatty acids

cell and may be determined in the medium (cf. e.g., GÄUMANN *et al.*, 1947). This method has been used more incidentally than for systematic investigations and it seemed attractive to study whether it could be made suitable for the comparative investigation of series of compounds.

The method adopted was as follows:

Cylinders (diameter 10 mm) were prepared from the beet by means of a suitable cork-borer and from these 10 disks (thickness 3 mm) were cut simultaneously by means of coupled razor blades. The disks were rinsed in distilled water until the colouring matter of the damaged cells had been removed, after which they remained in distilled water for one hour. After rinsing again to remove a small rest of free colouring matter, 10 disks were put into Erlenmeyer flasks containing 50 ml of a solution of the compound to be investigated. For one series the disks (100-120) from one beet were used and if some difference was observed as to the intensity of their colour, a number of comparable disks, equal to the number of flasks to be filled, were selected beforehand and of these one was put into each flask. The series consisted of different concentrations of the same compound, or of different compounds of the same (molar) concentration and were kept at 4-6° (in the refrigerator) or at room temperature.

The colouring matter leaving the cells was determined by measuring the colour intensity of the solution after suitable intervals by means of a photo-electric colorimeter (Colorimeter "Objecta", BLEEKER, Utrecht, equipped with monochromator and two photo cells) using green light (maximum 5000 Å), against blanks consisting of 10 disks immersed in the solvent only. The limit values for the absorption, reached when total discoloration occurred in a series, proved to be rather constant, indicating that the disks, selected as mentioned, always contained a fairly constant amount of colouring matter, as was shown also by running duplicates.

During the test (usually 22 hours) the blanks (in distilled water, sometimes in 12% alcohol) remained practically colourless or showed but a slight coloration.

It was observed that the pH in the mean shifted 0.5 units towards the acid side. In comparing

series of compounds simultaneously this effect causes no trouble, however. Buffer solutions could not be used, as their constituents influenced the test in an unfavourable way. In a further study of the beet test these observations will be analysed more in detail.

First of all the series of fatty acids, already studied with the oleate coacervate, was investigated (see Fig. 9).

Unfortunately this could not be done for the whole series, because of the fact that

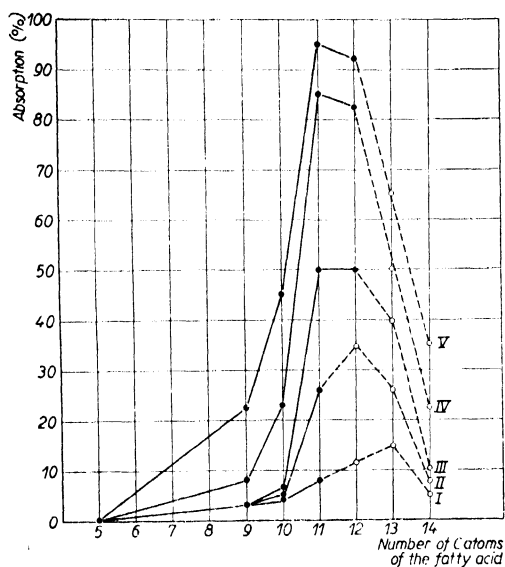


Fig. 9. Beet test. Normal fatty acids ( $C_6$ - $C_{14}$ ) I, II, III, IV, V: after 2.5; 4.5; 6.5; 12.5 and 22 hours respectively. ( $4 \cdot 10^{-4}$  mol/l. water,  $t = 20^\circ \text{C}$ ). Approximate measurements are indicated by open circles and dotted lines (cf. text).

the solubility of the higher homologues is too small at  $p_H$  7. From lauric acid ( $C_{12}$ ) onwards this causes opalescence or a slight turbidity of the solutions, interfering with the colorimetric determination, so that measurements at the beginning of the test in this region can only be approximative. Towards the end of the test the solutions become more and more clear as the acids are adsorbed into the beet tissue, but only for lauric acid this goes so far that the measurements become absolutely reliable. Though for this reason the interpretation of the curves has to be given with some caution, as micelle formation with the higher homologues may interfere, they nevertheless indicate that there exists a maximum in the same region as found with the oleate coacervate, so that this model system seems to react comparably with a biological object. And it can be concluded almost certainly already that the absence of a parallelism between the action of growth substances (derived from

indole or naphthalene) on the oleate coacervate and in the pea test (cf. the preceding paper) cannot be explained on account of the imperfection of the model system.

It would be of great importance if the form of the curves could be established accurately also for the higher numbers of the series. For, considered in connection with the conclusions arrived at by BOOIJ AND BUNGENBERG DE JONG (1949) in explaining the form of the curve for the action of fatty acids on the oleate coacervate, the occurrence of a maximum in the region  $C_{11}$ - $C_{12}$ , followed by a minimum at  $C_{15}$ - $C_{16}$  would indicate that the reacting constituent of the plasmic membrane is a system built in an orderly manner and that the length of its lipophilic part would be comparable with that of oleic acid.

It now becomes very interesting to determine the action of the growth substances in the beet test and to compare the effects with those on the oleate coacervate. The results are summarized in the Figs 10-13.

From these figures it can be deduced:

1. For naphthalene and indole derivatives the action on the membranes of the beet runs parallel to that on the oleate coacervate, as increasing hydrogenation, or

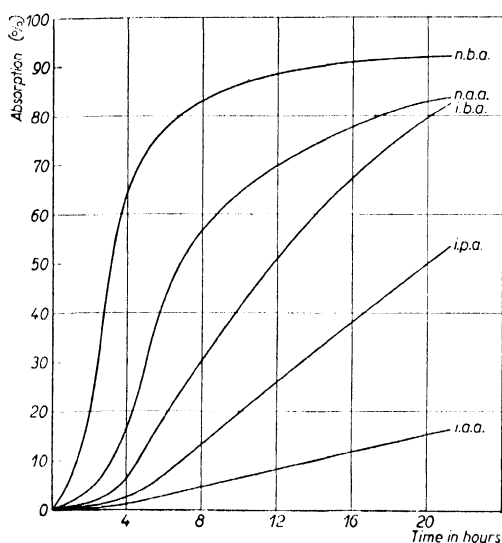


Fig. 10. Beet test: Indole acetic acid (i.a.a.); Indole propionic acid (i.p.a.); Indole butyric acid (i.b.a.); Naphthalene acetic acid (n.a.a.); Naphthalene butyric acid (n.b.a.); ( $10^{-2}$  mol/l; 12% alcohol,  $t = 4^{\circ}\text{C}$ )

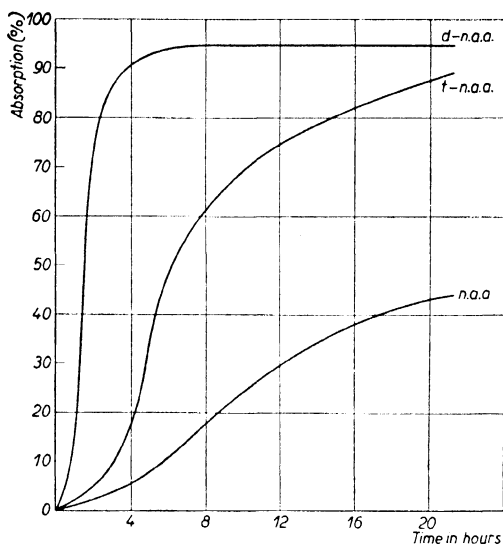


Fig. 11. Beet test: Naphthalene acetic acid (n.a.a.) and its tetrahydro- (t-n.a.a.) and decahydro- (d-n.a.a.) derivative ( $10^{-2}$  mol/l; water,  $t = 4^{\circ}\text{C}$ )

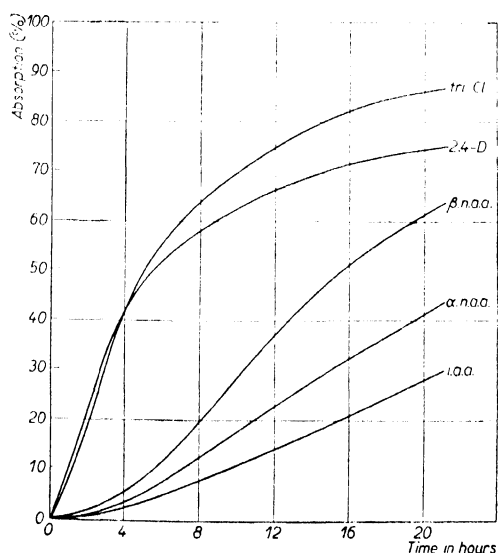


Fig. 12. Beet test: Indole acetic acid (i.a.a.);  $\alpha$ -Naphthalene acetic acid ( $\alpha$ -n.a.a.);  $\beta$ -Naphthalene acetic acid ( $\beta$ -n.a.a.); 2,4-Dichlorophenoxy-acetic acid (2,4-D); 2,4,6-Trichlorophenoxy-acetic acid (tri.Cl); ( $2.5 \cdot 10^{-3}$  mol/l; water;  $t = 18^{\circ}\text{C}$ )

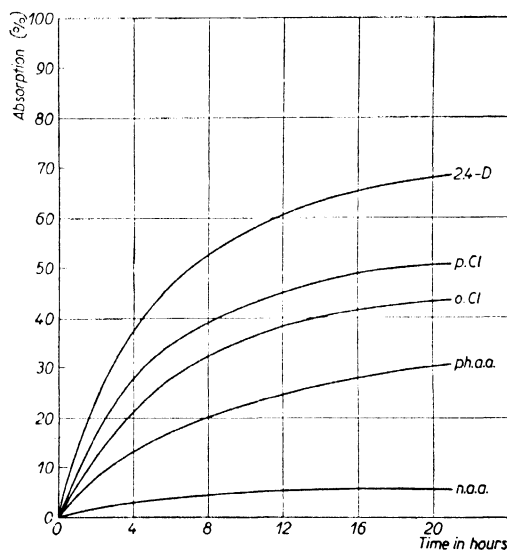


Fig. 13. Beet test: Naphthalene acetic acid (n.a.a.); Phenoxy-acetic acid (ph.a.a.); o-Chlorophenoxy-acetic acid (o.Cl); p-Chlorophenoxy-acetic acid (p.Cl); 2,4-Dichlorophenoxy-acetic acid (2,4-D); ( $2.5 \cdot 10^{-3}$  mol/l; water,  $t = 4^{\circ}\text{C}$ )

lengthening of the side chain results in increasing liberation of the colouring matter.

2. The higher the activity of these compounds in the pea test (plant growth activity), the weaker their effect on the beet membranes.

3. The action of indole derivatives in the beet test is decidedly weaker than that of the comparable naphthalene compounds, on the other hand it is a well-known fact that especially with the butyric acids the indole derivative exhibits the strongest growth activity.

4. The activities of the different compounds in the beet test reflect very well their phytotoxicity as already known from the study of their effects on parts of plants or intact plants (rooting of cuttings, inducing parthenocarp, etc.). This is very evident if one compares indoleacetic acid < naphthalene acetic acid < 2,4-dichlorophenoxyacetic acid.

5. Phenoxy-acetic acid, and its chlorinated derivatives on the whole, affect the membranes more strongly than *e.g.*, naphthalene acetic acid.

In this series not only the actions on the coacervate (Fig. 14) and in the beet test run parallel, but—in contrast with that of naphthalene and indole derivatives—the physiological activity (exhibited at low concentrations) also increases in the same sequence. Moreover their herbicidal effects (in higher concentrations) show the same course.

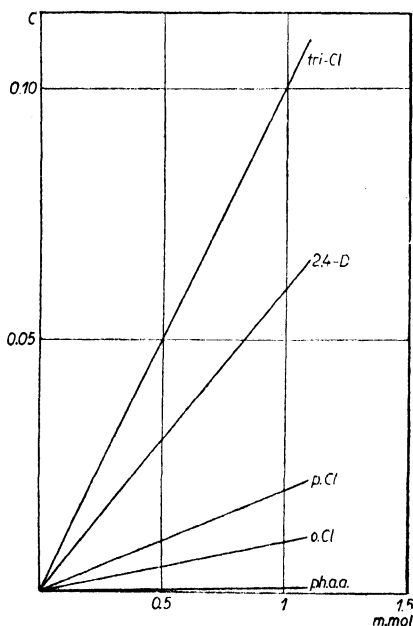


Fig. 14. Oleate coacervate. Shifting of the KCl-curve (ordinate) under influence of phenoxy-acetic acid (ph.a.a.), o.chlorophenoxy-acetic acid (o.Cl), p.chlorophenoxy-acetic acid (p.Cl), 2,4-dichlorophenoxy-acetic acid (2,4-D) and 2,4,6-trichlorophenoxy-acetic acid (tri.Cl)

These results give rise to the following considerations:

In the series of naphthalene acetic acid and its hydrogenated derivatives or naphthalene and indole acetic acid and their homologues the highest physiological (growth) activity is found at the beginning of the series, indicating that here the proper balance between lipophilic and hydrophilic parts of the molecule, required for maximal activity, has already been reached. Increase of the lipophilic character, by hydrogenation of the nucleus or lengthening of the sidechain, causes stronger interaction with (adsorption to) the protoplasmic membranes and on the whole a diminished growth activity.

Starting with the weakly active growth substance phenoxy-acetic acid with too weak a lipophilic character, increase of the lipophilic part (by introduction of chlorine atoms) is followed by increasing interaction with the membranes, but now also by an increase of the growth activity up till 2,4-dichlorophenoxy-acetic acid, where the

proper balance is reached. For, if one more chlorine-atom is introduced, the activity of the resulting 2,4,6-trichlorophenoxy-acetic acid in the pea test proves to be greatly

diminished (cf. Fig. 6) whereas the activity in the coacervate as compared to that of 2,4-dichlorophenoxy-acetic acid has still increased. See Fig. 14.

So from the dichloro acid onwards the same divergence between biological object and model system is encountered as that found with the naphthalene and indole series discussed above.

In the same sense it can be understood that chlorination of naphthoxy-acetic acid to 2,4-dichloronaphthoxy-acetic implies an "overpowering" of the lipophilic "weight" and consequently together with increasing interaction with the coacervate a diminished growth activity.

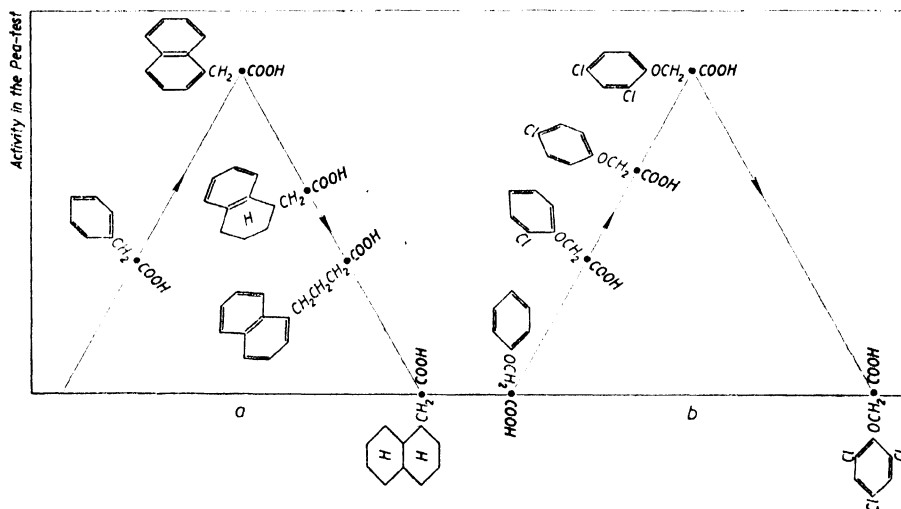


Fig. 15. Schematic representation of the balance between hydrophilic and lipophilic parts of the molecules in the series: A. Phenyl acetic acid, naphthalene acetic acid and its hydrogenated derivatives and homologues; B. Phenoxy acetic acid and its chlorinated derivatives.

The HL-balance is brought into relation with the physiological activity (in the pea test). The most favourable value in this sense (maximal activity) is indicated by a horizontal position. If the hydrophilic carboxyl group or the lipophilic part dominates, the position becomes an inclined one, with as extremes vertical positions (physiologically inactive), caused either by total dominance of the carboxyl group (compound is too water soluble) or of the lipophilic part (compound is too fat soluble).

Most likely the conclusion arrived at by TEMPLEMAN AND SEXTON (1946) in their studies on the differential effect of synthetic plant growth substances: "chlorination of the phenoxy compounds generally appears to increase their activity, whereas this is not so for chlorination of the naphthoxy compounds", can be explained on the same grounds.

In our opinion all these facts fit into the same scheme, the value of the balance between lipophilic and hydrophilic parts of the molecules being the decisive magnitude (cf. VELDSTRA, 1949). And it wholly depends on the starting points whether parallelism for activities with the biological object (pea test) and in the model system (oleate coacervate) is observed or not.

These relations are represented once more in a schematic form in Fig. 15.

We believe that from this point of view also the higher physiological activity of indole butyric acid as compared to that of naphthalene butyric acid may be understood. Of these two ring systems the indole nucleus has a considerably weaker lipophilic character than naphthalene (derived from the difference between their condensing

actions in the oleate coacervate; compare also HAVINGA, VELDSTRA, 1948). For this reason the size of the lipophilic side chain can be somewhat longer when attached to indole than when coupled with naphthalene before the maximum balance with regard to the hydrophilic carboxyl group is reached, and thereafter the physiological activity decreases. Of course we realize that in these considerations another important factor is neglected, namely the influence of *e.g.*, introduction of chlorine atoms or of lengthening of the side chain on the position of the carboxyl group with respect to the ring system. Before *e.g.*, dipole moments of suitable derivatives will have been determined this factor cannot be analysed further, however.

The importance of the HL-balance of an amphipatic compound to its behaviour in a certain system could be clearly demonstrated in the series of di-alkyl acetic and di-alkyl malonic acids respectively, by determining their effect in the oleate coacervate (see Fig. 16).

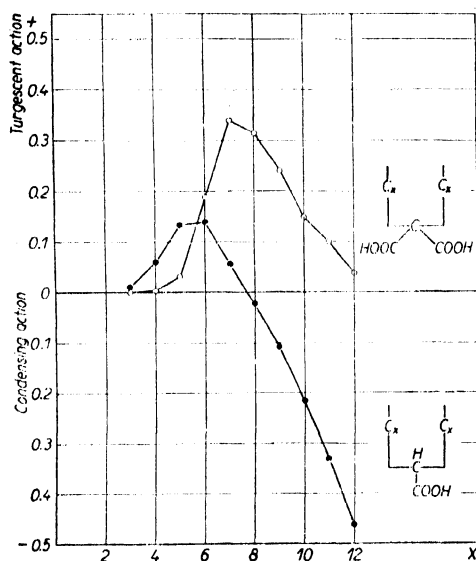


Fig. 16. Oleate coacervate. Shifting of the KCl-curve (ordinate) under the influence of di-n-alkyl acetic acids (●—●) and of di-n-alkyl malonic acids (○—○) (concentrations:  $5 \cdot 10^{-4}$  mol/l)

the acetic acids, but has shifted quite marked'y to the swelling side and to the right. This means that at the same value of  $x$  (for  $x \geq 6$ ) the swelling effect of the malonic acids is stronger than that of the acetic acids and that the influence of increasing lipophilic character becomes perceptible "later", *viz.*, at a higher value of  $x$ . Obviously this is caused by the introduction of another carboxyl group, by which the hydrophilic "weight" is doubled.

Quite clearly the action in the oleate, both in a quantitative and in a qualitative sense, is governed by the HL-balance of these acids.

If one tries to imagine why increasing the lipophilic character of a compound beyond a certain limit causes a decreasing physiological activity, in our opinion the results obtained with the beet test may offer a plausible explanation, namely that the

interaction with (adsorption to) the protoplasmic membranes then becomes too strong. If indeed, as already suggested in the preceding paper, the primary reaction takes place in the protoplasm, too strong an adsorption to the membranes might prevent the attaining of the concentration (within the protoplasm) required for maximum activity. Furthermore too strong an interaction, with as a possible consequence, too strong an opening effect on the membranes might disturb the normal (physiological) equilibrium between the protoplasm and the outside of the cell. It is even imaginable that the latter effect is partly responsible for the herbicidal action of 2,4-dichlorophenoxy-acetic acid (for this purpose used in concentrations up till  $5 \cdot 10^{-3}$  mol/l), which compound, as shown in the beet test, has a strong affinity for the membranes. In that case the well-known selectivity of this action, as expressed by the far more greater toxicity for dicotyledonous than for monocotyledonous plants, a.o. might be connected with differences in composition and consequently in reactivity of the plasmic membranes. As to the selectivity in action of this type of compounds we truly are inclined to attribute quite generally decisive importance to the relation between H<sub>L</sub>-balance of the active compound on the one side and the composition of the system on the other side (cf. also VELDSTRA, 1944, page 154 under 3).

It now also becomes necessary to revise the view, expressed previously, concerning the inhibiting action of growth substances, if applied in excess, namely that this would be caused by a condensing action on the membranes. In the oleate coacervate only opening actions were observed, secondly they never changed to condensing ones when the concentration of the growth substances was increased. Therefore, also in connection with the experience in the beet test, it seems more likely now that in the inhibition at higher concentrations rather too strong an opening action plays a rôle. On a closer examination this is more plausible too, when considered from a colloid chemical point of view. Of course this does not exclude the possibility that in the growth inhibition by other types of compounds, such as the neutral coumarin or related lactones, such condensing actions are of importance. More probably, however, as with the growth stimulating action, the inhibiting one can neither be explained solely by these membrane effects. This appeared from the fact that the blastocholine activity in the series of indole- or naphthalene acetic acid and its hydrogenated derivatives decreases just like the growth activity by lengthening of the side chain or by increasing hydrogenation.

And so we must conclude that the view expressed before concerning the localization of the action of the growth substance has certainly been connected too one-sidedly with the membranes.

According to the results obtained with the oleate coacervate and in the beet test the interaction with the membranes may certainly occur and, moreover, is of the type that answers expectations. But it is quite certain now that in the starting hypothesis concerning growth substance action the importance of this interaction has been overestimated and that it does not constitute the "primary reaction" itself, which will more probably be found in the cytoplasm. The membrane effect, however, can influence this primary reaction in a quantitative sense and will be responsible to a large extent for the difference in activity in a series of related compounds. In this way it implies a form of selectivity.

This view leads to the following considerations:

*Case A.* Of the amount of a highly active growth substance (naphthalene acetic



acid) added to a biological system part will be adsorbed to the endo- and ectoplasmic membranes (and possibly onto other interfaces outside the cytoplasm) and part will act within the cytoplasm (primary growth reaction), both parts being in dynamic equilibrium with each other and with the molecules present in the vacuole and outside the cells.

*Case B.* If to the same system be added a derivative of naphthalene acetic acid with increased lipophilic character, and consequently of higher interface activity (*e.g.*, naphthalene butyric acid or decahydro naphthalene acetic acid) in the same molar concentration, within a certain course of time a greater part will be adsorbed to the plasmic membranes, etc. Therefore a smaller amount of molecules will be available for the primary reaction, causing a weaker growth-response than in the case of A. The stronger the affinity for the membranes, the weaker the growth effect will be (naphthalene butyric acid: weakly active, decahydro naphthalene acetic acid: practically inactive).

If this schematic representation touches the essence of the growth substance action, it should be possible:

1. To obtain the same growth effect as in A with a lower concentration of naphthalene acetic acid, if a compound as used in B is added too. Because of its higher interface activity the B-compound will be preferentially adsorbed to the membranes and consequently of the lesser amount of naphthalene acetic acid (as compared to that in A) enough still remains available for the primary reaction. The B-compound will be the more effective in this sense, as its growth activity is lower.

Such a mixture of "underdosed" highly active growth substance and a supplementary quantity of a weaker active or nearly inactive compound should equal the maximum effect of the growth substance alone, even if the total number of molecules is smaller, as for a comparable "occupation" of the membranes, etc., a lesser amount of a higher interfacial activity compound will be sufficient.

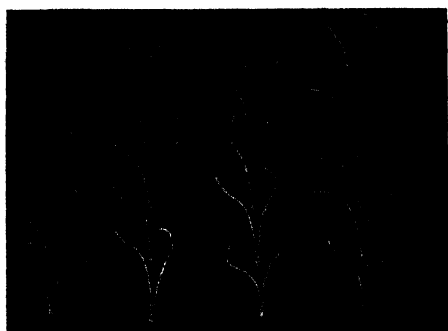
2. The activity of a weakly active substance as mentioned in Case B (weakly active because of too strong a lipophilic character) should be enhanced in the presence of a related compound with still higher interface activity. The first-mentioned substance then will be displaced from its adsorbed state and become available to a higher degree for the primary reaction. (The proportion of the interface activity can be derived from the results obtained with the oleate coacervate or in the beet test).

Both suppositions could be substantiated by assaying mixtures of the required composition in the pea test (*cf.* Figs 17, 18, 19).

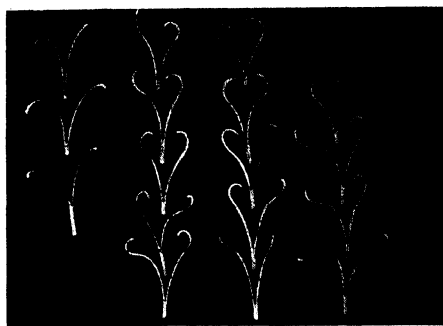
So it proves to be possible to obtain a maximal effect with a sub-maximal concentration of *e.g.*, naphthalene acetic acid by the addition of the less active naphthalene butyric acid or of the inactive decahydro naphthalene acetic acid in quantities which exhibit only a very weak or no activity of their own. Even if the concentration of naphthalene acetic acid is lowered to one tenth or one fourtieth of that normally used, maximum activity can be attained. Fig. 18 shows that maximal activity can be reached with half the quantity of the molecules required for naphthalene acetic acid if used alone.

The normal fatty acids of which the molecular size corresponds with that of naphthalene acetic acid [as *e.g.*, undecanoic acid (XXIII) and dodecanoic acid (XXIV)] also show this enhancing effect, though to a less extent.





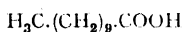
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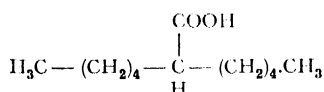
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Fig. 19. Pea test. Synergistic actions.

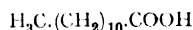
1. Naphthalene acetic acid, from 1. to 1.: 4; 1; 0.4;  $0.1 \cdot 10^{-5}$  mol/l  
 2. Naphthalene acetic acid, " " " " " " :  $\left\{ - \left\{ 1 \right\} \left\{ 0.4 \right\} \left\{ 0.1 \right\} \cdot 10^{-5} \text{ mol/l} \right.$   
 Di-n-amylacetic acid, " " " " " " :  $\left\{ 4 \right\} \left\{ 4 \right\} \left\{ 4 \right\} \left\{ 4 \right.$



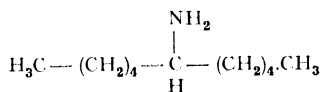
XXIII



XXV



XXIV



XXVI

If the carboxyl group is moved towards the centre of the carbon chain, and the molecules of the fatty acid thus assume the form known to be required for maximal activity as a wetting agent or penetrant (WILKES, WICKERT, 1937; HARTLEY, 1941;

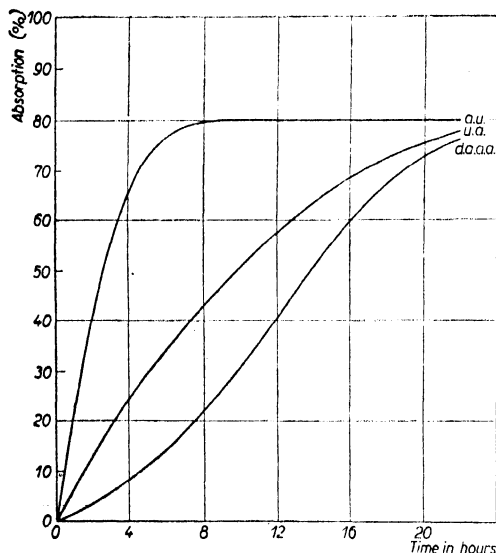


Fig. 20. Beet test. Di-n-amylacetic acid (d.a.a.).  
 Undecanoic acid (u.a.); 6-Amino-undecane (a.u.)  
 ( $8 \cdot 10^{-4}$  mol/l; water;  $t = 20^\circ \text{C}$ )

PRICE, 1946), the resulting di-n-amylacetic acid (XXV) proves to be a very potent activator, (cf. Figs 17, 19), though its activity in the beet test is even weaker than that of undecanoic acid (cf. Fig. 20) and its activity therefore cannot be explained by an unusually enhanced affinity for the membrane.

So with these fatty acids, which—in the concentrations used—are completely inactive as growth substances, the property to act as synergists seems to be connected with a certain spatial structure, in a certain sense quite comparable with that derived previously for the highly active growth substances proper.

In fact, di-n-amyl-acetic acid is a highly active “internal” wetting agent in relatively low concentrations for e.g., pea stem tissues, as these rapidly become transparent when being embedded in a

solution of it. The uptake of water under the influence of compounds of this type is at the moment the subject of a separate investigation.

In this connection the question arose quite logically whether wetting agents in general, if showing high activity in the beet test, would be able to enhance the activity of sub-maximal concentrations of growth substances. This proved not to be the case, *e.g.*, for the types mentioned on page 280.

It was very interesting to find that 6-amino-undecane (XXVI) which is still more active in the beet test than the corresponding acid (cf. Fig. 20) does not show the enhancing effect either (cf. Fig. 21). So the carboxyl group, as with the growth substances

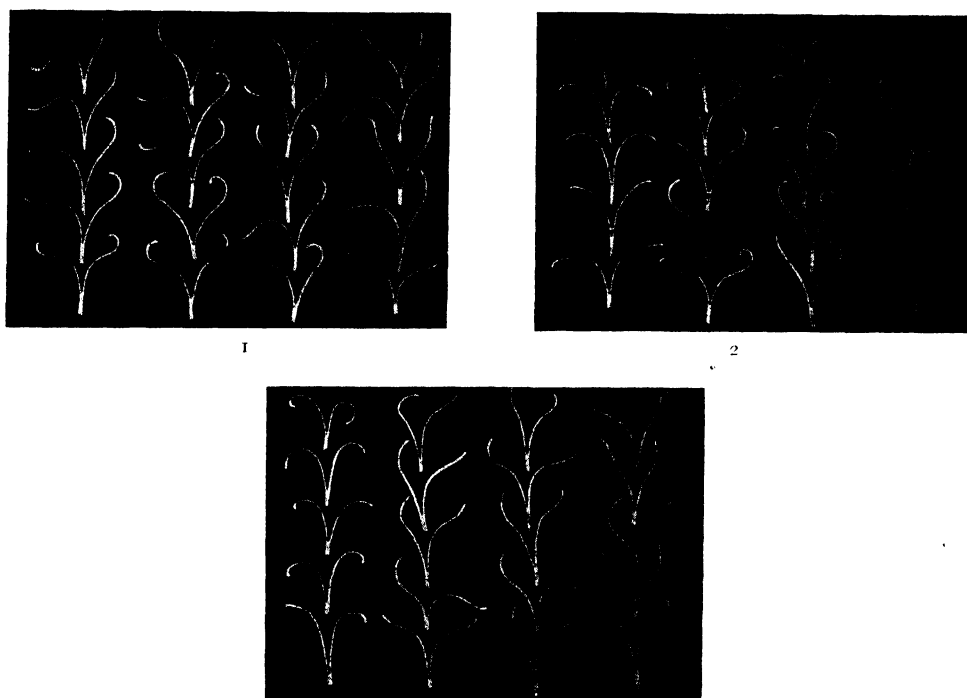


Fig. 21. Pea test. Synergistic actions.

1. Naphthalene acetic acid, from 1. to r.: 4; 1; 0.4; 0.1 · 10<sup>-5</sup> mol/l
2. Naphthalene acetic acid, .. .. . : { - { 1 { 0.4 { 0.1 · 10<sup>-5</sup> mol/l  
    Di-n-amyl-acetic acid, .. .. . : { 4 { 4 { 4
3. Naphthalene acetic acid, .. .. . : { - { 1 { 40. { 0.1 · 10<sup>-5</sup> mol/l  
    6.Amino-undecane.HCl, .. .. . : { 4 { 4 { 4

proper, apparently has a specific function. The results of investigations with several types of carboxylic acids will be published in a separate paper. In connection with the present discussion we will only mention that it was found that acids generally show the enhancing effect if, as compared with the highly active growth substances, the lipophilic character has increased and the molecular size is of the same order\*.

It is interesting in this connection that recently THIMANN and BONNER (1948) reported about the increase of growth promoting action of low concentrations of indole

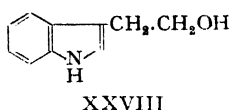
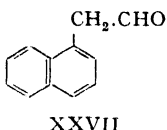
\* Patent application pending.

acetic acid by means of tri-iodo benzoic acid, practically inactive itself. The effect is large in the pea test, but smaller in the straight growth of *Avena* coleoptiles and in the standard *Avena* test. We will discuss these results more extensively in the above-mentioned paper.

It will be attractive to study whether this type of synergistic action of structural analogues will be of importance in other cases, also because of the fact that up till now antagonistic effects have been emphasized practically exclusively in the study of the action of structural analogues (cf. VELDSTRA, 1948).

When surveying once more the data obtained in the present investigations one arrives in our opinion at the conclusion that in the action of growth substances two phases can be distinguished, namely one concerned with adsorption to protoplasmic membranes, etc., and one constituting the primary growth reaction proper, most probably proceeding within the protoplasm.

For the latter supposition further arguments were found in the behaviour of the neutral compounds naphthalene acetaldehyde (XXVII) and tryptophol (XXVIII) in the pea test.



The aldehyde proved to be nearly as active as naphthalene acetic acid, whereas the alcohol only showed a small effect (cf. LARSEN, 1947, who already investigated these compounds in the *Avena* test). But in both cases the action was distinctly more rapid than that of naphthalene acetic acid. If an essential part of the growth reaction does take place inside the cell, this difference in reaction velocity may be explained in a plausible way, namely by a more rapid permeation of the neutral compounds as compared to that of the analogous acids. (Inside the cells both compounds are very probably oxidized to the acids).

As to the membrane effect, from the results with the oleate coacervate and also with the beet test it can be deduced that in a physico-chemical sense the growth substances are apt to exert an "opening" action on lipophilic membranes, but under the present conditions we cannot state definitely whether this really occurs in the physiologically active concentration region to the extent that this adsorption performs a physiological function or whether this adsorption properly speaking must be considered as a "waste" with respect to the total growth reaction. The fact that, as mentioned above, such structural details as "wetting agent type" are apparently of importance for the synergistically active acids, makes us inclined for the present to the supposition that indeed the membrane effect is of physiological importance and that the growth reaction thus proceeds in two phases. From the proportions found with the activity of mixtures of growth substances and synergistically active acids it must be deduced then that by far the greater part of the growth substance is used in the first phase and only a small fraction is actually required for the primary reaction.

In our opinion it seems very likely that the first "phase" of the growth reaction, as deduced in this way, is the same as that indicated as early as 1939 by F. W. WENT as the preparatory reaction when he proved that pre-treatment of the objects in the *Avena*

or pea test with *e.g.*, phenyl butyric acid or cyclohexane acetic acid (not active in the elongation reaction itself) increased the response of subsequently applied indole acetic acid. The compounds active in this respect were called hemi-auxins. In comparison with the compounds investigated by us phenyl butyric acid and cyclohexane acetic acid are but very weakly active, however, as we determined by using phenyl butyric acid in our tests.

(Cf. also the concentration used by WENT for phenyl butyric acid in the pea test:  $\sim 10^{-3}$  mol/l, whereas *e.g.*, di-n-amyl-acetic acid acts with  $10^{-6}$ – $4 \cdot 10^{-5}$  mol/l).

It was stated by WENT that indole acetic acid is active both in the preparatory and in the elongation reaction, but that the former reaction requires a higher concentration than the latter. So here the proportions are comparable to those we found in the pea test with the afore-mentioned mixtures of naphthalene acetic acid and a synergist.

Furthermore according to WENT the preparatory reaction ("first phase") is insensitive to the  $p_H$ , whereas the primary reaction itself ("second phase") is dependent on it. These relations could be understood if indeed the first phase is concerned in the main with the protoplasmic membranes, *viz.*, with the outside of the cells, because for such an action one would not expect a great difference at a different  $p_H$ . As the membrane has to be passed for the primary reaction (inside the cell), the degree of dissociation of the acids plays a rôle and consequently the  $p_H$  of the medium will exert a pronounced influence.

In their important study on the interactions of growth substances in growth and inhibition, SKOOG, SCHNEIDER, AND MALAN (1942) did not agree with WENT's conclusions, as in the *Avena* test and in the cylinder test (straight growth) inhibiting effects were mainly observed if phenyl butyric acid was added to indole acetic acid; these effects were ascribed to a competitive action between the acids. Only in the cylinder test with low concentrations of indole acetic acid (0.005 mg/l) and a relatively high concentration of phenyl butyric acid a synergistic effect was observed, explained by SKOOG *et al.* as an auxin-sparing action of phenyl butyric acid.

With higher concentrations of indole acetic acid the initially occurring antagonistic effect (at low concentrations of phenyl butyric acid) is only partially compensated by increasing the concentration of phenyl butyric acid (cf. Fig. 2 of SKOOG *et al.*, reproduced in Fig. 22).

For this reason SKOOG *et al.* concluded that in this case it is impossible to interpret the effects in terms of hemi-auxin activity of phenyl butyric acid, as then the growth response of the mixtures should equal the optimal action of indole acetic acid itself.

We deem it possible, however, that this

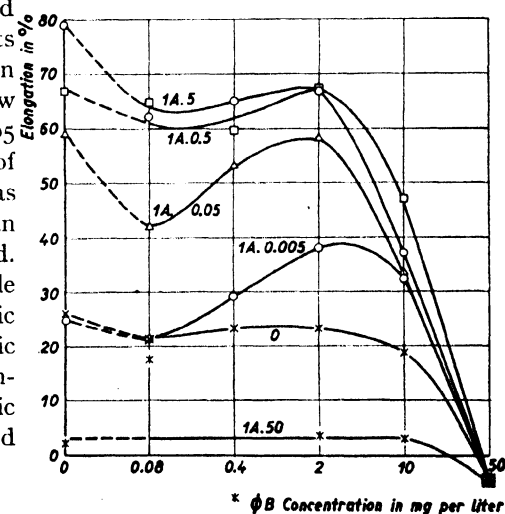


Fig. 22. Reproduction of Fig. 2 and legend according to SKOOG, SCHNEIDER, AND MALAN (1942). Per cent elongation of 3 mm long sections of *Avena* coleoptiles in the section test with 2% sucrose solution and various concentrations of indole acetic and phenyl butyric acids. (The numbers inserted represent concentrations of indole acetic acid in mg/l).

optimal level is not reached because of the fact that with phenyl butyric acid—only weakly active as a synergist in our test—the toxic concentration is interfering too soon, and it remains to be investigated if under the circumstances of the test of SKOOG *et al.* the acids highly active synergistically described by us would not meet the requirements for hemi-auxins.

For the rest it seems to us that the course of the curves does not procure arguments for a competitive action as discussed by SKOOG *et al.*, as *e.g.*, the relative inhibition caused by 0.08 mg phenyl butyric acid/litre is smaller for indole acetic acid 0.5 mg/l than for indole acetic acid 5 mg/l. For a truly competitive antagonism this would have to be the reverse and moreover it cannot be understood how by increasing the concentration of the “antagonist” phenyl/butyric acid the inhibition is overcome. In the relation metabolite/metabolite-antagonist it is only known that at very low concentrations of the antagonist, a stimulating action may occur (cf. HENRY, 1943; WOOLLEY, WHITE, 1943; WOOLLEY, 1944) and that the antagonistic effect increases with increasing concentration, which is just the reverse of what is observed in SKOOG’s test.

In our opinion the apparently complex relations in the action of mixtures in SKOOG’s experiments have not yet been satisfactorily unravelled. The discussion of these interesting observations can be pursued after the synergists, which we have described, have also been investigated.

For the moment we prefer to follow the “route” of a two-phase growth reaction, the more so as in one of the most important contributions to the investigations concerning the mechanism of action of plant growth substances BURSTRÖM (1941, 1942, 1945, 1947) has presented convincing evidence that the cell elongation (studied with individual wheat root cells) proceeds in two distinct phases. During the first phase the primary wall is loosened, possibly no new material being added, accompanied by increasing wall elasticity and turgor tension. This phase is highly temperature-sensitive, acceleration following increase of the temperature. Nutritional conditions, however, prove to be of little importance.

In the second phase the rate of cell elongation—slow until then—increases very much, so that two-thirds or more of the total increase in cell lengths takes place in a short time. The turgor tension now remains constant, whereas the elasticity decreases. This second phase, during which oriented cellulose strands are deposited in the wall, is strongly influenced by the nutrient supply; *e.g.*, carbohydrate supply causes an extension.

As to the influence of indole acetic acid it appeared that it accelerates the first phase, but that the second phase is more or less completely inhibited. If very low concentrations of indole acetic acid are used, the retardation of cell elongation, caused by this influence on the second phase, is followed by a stimulation of the elongation.

It now becomes most interesting to investigate whether the “phases” deduced from the more (physico-)chemical analysis of the problem have something to do with those distinguished by BURSTRÖM. In this connection one will first of all have to determine whether the acids, active synergistically in the pea test, have some typical action on the first and/or the second phase, as described by BURSTRÖM. It will depend on the outcome of these investigations\* in which direction the discussions will have to be pursued.

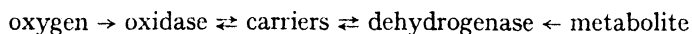
\* Prof. BURSTRÖM was so kind as to accept our proposal that these questions be studied in his laboratory at a convenient time.

Before we terminate our considerations by giving some suggestions concerning the mode of action of the growth substances in the cytoplasm in connection with the information gathered from our experiments, it seems useful to survey briefly the views about growth substance action put forward until now by different investigators and to analyse in what respects these views converge.

As for the growth response by the natural auxins oxygen and carbohydrate are indispensable and as there is evidence that auxin action is in some way connected with respiration (see below) it is quite comprehensible that there still exists a very definite trend to deal with the auxins under the heading "co-enzymes". This means incorporation of the auxins in the ergons, mainly belonging to the vitamin B group, of which it is known with certainty that they form part of prosthetic groups of redox enzyme systems (cf. *e.g.*, the review by SKOOG, 1947, pages 542–544). SKOOG even goes so far as to suggest that, because of the aerobic nature of auxin action, together with the indispensability of the double bond in the auxins and its exact position in the ring, the reversible saturation of this double bond may simply constitute the specific reaction of the auxin molecule.

From a chemical point of view this is highly improbable, however, as the experience with biochemical hydrogenations (*e.g.*, with yeast, cf. FISCHER, 1939) has shown that hydrogenation of the double bond is difficult when connected with a tertiary C-atom (both in aliphatic chains and in hydro-aromatic ring-systems) and that  $\alpha$ ,  $\beta$ -unsaturated alcohols are not hydrogenated, in contrast to the primary ones. And even if the dihydro-auxin could be formed under physiological conditions, with this type of compounds it is rather certain that this hydrogenation would not be reversible (required for the supposed physiological function) as oxidation of a cyclopentane ring-system to a cyclopentenoderivate is quite unknown under these conditions. The vitamins functioning in a redox-system and all compounds which can replace them to a certain extent (*e.g.*, methyleneblue, etc., with low negative redoxpotentials) are of a fundamentally different structure and from a chemical point of view there is little doubt that neither the auxins  $\alpha$  and  $\beta$ , nor indole acetic acid and the synthetic compounds with growth substance action possess the redox-character required for functioning in this sense under physiological conditions. Also by studying the synthetic compounds polarographically it has already appeared that they are not easily hydrogenated (VELDSTRA, 1944). So in our opinion the "co-enzyme" conception in the chemical sense will have to be abandoned and we see more perspectives in trying to link in another sense the physico-chemical type of action—as deduced by one of us from the chemical and spatial structure of the growth substances—to the results of investigations by *e.g.*, COMMONER, THIMANN (1941), AVERY *et al.* (1943–1944), which indicate that auxins play a rôle in the relation growth/respiration. In 1941 COMMONER and THIMANN raised the question anew whether there exists some relation between growth and respiration. They were unsatisfied by the conclusions of former investigations (KÖGL *et al.*, 1936; BONNER, 1936), implying that auxin does not have an effect on the respiration of *Avena* coleoptiles, whereas to their opinion many facts point to the existence of some relation.

Considering 1st that inhibition of respiration by cyanides—which in the chain:



is concerned with the oxidase—causes a proportionate inhibition of growth, 2nd that, also in non-growing tissues respiration does occur, COMMONER AND THIMANN concluded

*References p. 311/312.*



that the differentiation of the two processes had to be looked for in the dehydrogenase part of the chain. For this reason they studied the effect of dehydrogenase inhibitors on growth and found it to be an inhibiting one.

Particularly iodoacetate proved to inhibit growth of *Avena* coleoptiles in solutions containing sucrose and indole acetic acid. This inhibition of growth is complete with an iodoacetate concentration of  $5 \cdot 10^{-5}$  mol/l whereas under these conditions the respiration is only inhibited by 10%. This part of the respiration then could be essentially linked to the growth process. The effect of iodoacetate could be removed by the four-carbon acid-ions malate and fumarate and to a lesser extent by succinate and pyruvate. These acids may function as carriers in the chain indicated above. The acids reinforce the effect of indole acetic acid on growth and also the respiration if indole acetic acid is present. After pre-treatment of the coleoptile sections with malate or fumarate the respiration of these objects is stimulated by indole acetic acid, without such a pre-treatment it has no effect and in this respect the results of the former investigations are confirmed. As under these conditions the influence of indole acetic acid on respiration and the effect on growth parallel each other, COMMONER AND THIMANN deduced that the  $C_4$  acids are involved in a respiratory system essential for growth, which constitutes only a small fraction of the total respiration. This particular respiratory process was considered to be promoted by indole acetic acid.

ALBAUM AND COMMONER (1941) and ALBAUM AND EICHEL (1943) found similar relations for growth inhibition by iodoacetate and its reversal by  $C_4$  acids with intact oat seedlings.

Recently THIMANN AND BONNER (1948) repeated the experiments more in detail and could confirm the previous results. Besides the  $C_4$  acids already mentioned also citrate, malonate and maleate proved to be able to reverse the inhibition by iodoacetate. Growth of isolated sections in a solution containing indole acetic acid ( $5.7 \cdot 10^{-6}$  mol/l) and sucrose ( $3 \cdot 10^{-2}$  mol/l) proved to be markedly increased if the sections were only partly submerged or by oxygenating submerged sections. Obviously the aerobic condition is of great importance for the processes involved, and as shown by the experiments, primarily in the presence of indole acetic acid. The remarkable fact was established that the sensitivity, both to iodoacetate inhibition and to the growth promoting action of the organic acids, augments with increasing age of the coleoptile from which the sections are cut. The authors suggest that growth is controlled by an enzyme containing free SH-groups, essential for its activity. The concentration of iodoacetate required to effect growth inhibition is presumed to be a measure for the concentration of the enzyme. According to the authors the amount of this enzyme (per unit length of the coleoptile) would then decrease with increasing age and its effective concentration would be enhanced by oxygen. As to the latter conclusion it seems difficult to us to imagine how the effective concentration of an enzyme, for the action of which free SH-groups are essential, can be enhanced by oxygen supply. And we are inclined to ask whether it would not be more plausible to suppose that oxygen is required for the functioning of the enzyme leading to a growth response. Though of course many other questions still remain unanswered these clear-cut experiments will certainly contribute a great deal to the solution of the problem of growth substance action.

The more so if they are connected with the investigations of BERGER AND AVERY (1943, 1944) who studied the influence *in vitro* of synthetic growth substances on dehydrogenase systems of the *Avena* coleoptile. The action of cell-free extracts containing

these enzymes was not influenced by growth substances, but if the enzyme extracts were prepared from coleoptiles, pre-treated with indole acetic acid, particularly the action of the extract containing the alcohol dehydrogenase was enhanced as compared with that of the controls. For other dehydrogenases a similar effect was not found or only to a lesser degree. This is a very interesting observation, as among the dehydrogenases the alcohol dehydrogenase is by far the most sensitive to iodoacetate. Thus the authors are led to consider auxin action as an activation of an enzyme. As activation has to be relative to a constant amount of the enzyme and it is not certain that the conditions of the experiment meet this requirement (in our opinion an enhanced extractivity of the enzyme may play a rôle as well) this conclusion, though highly alluring, cannot yet be definite on account of these experiments. It seems to us, however, that continuation of this type of experiments is of the utmost importance to the study of growth substance action. Particularly as in this way we may expect to receive an answer to the question whether growth substances are concerned with the energy consuming processes of the uptake of sugar and water by the cells. The question was put in this way by FREY-WYSSLING (1947) in a discussion on the biochemistry of cell elongation (cf. also FREY-WYSSLING, 1945), after having given as his opinion that during elongation not only the intake of sugar (opposite to the diffusion gradient and therefore requiring energy (ARISZ, 1939, 1945), supplied by the respiration) but also that of water takes place with consumption of energy. In this connection it will be of great importance to follow FREY-WYSSLING's advice not to relate respiration to coleoptiles or cells but to the quantity of protoplasm, as the cell walls and the contents of the vacuole do not consume oxygen.

This question greatly interests us as the peculiar relation between the structure of the growth substances (particularly also in a spatial sense) and their activity as deduced from our investigations made us consider them apt to perform a function related with water- and sugar-uptake. Certainly this view was at first connected too one-sidedly with membrane effects, but also for the action taking place in the cytoplasm one has to ask whether this relation structure/activity may procure a lead.

There are already many indications from other investigations that growth substance action is in some way or another related to the uptake of water and sugar by the cells. COMMONER *et al.* (1942, 1943) studied the absorption of water by potato slices and found that this was stimulated by indole acetic acid, as already stated before by REINDERS (1938). This led to the conclusion that the effect of growth substances on cell elongation largely consists of a regulation of water absorption, probably by influencing the adsorption of osmotically active salts.

VAN OVERBEEK (1944), criticizing these experiments because of the fact that they were not performed under aseptic conditions and because REINDERS (1938) had already found that increased uptake of water also occurs in distilled water, repeated them, taking these facts into consideration. Also under aseptic conditions indole acetic acid and naphthalene acetic acid proved to induce increased uptake of water, both in distilled water and in mannitol or sucrose solutions.

As the expressed sap of tissues treated with growth substances (the method used implies that the cells are crushed) possessed a lower osmotic concentration than that of non-treated tissues, VAN OVERBEEK concludes that the enhanced water uptake of the cells induced by growth substances can be due to a decreasing wall pressure only or to an increase in non-osmotic water uptake, or to both.

*References p. 311/312.*

SHOWACRE AND DUBUY (1947) arrived at similar conclusions in studying the relation of water availability and growth substances in the growth of *Avena* coleoptiles. One of the most interesting results is that although oxygenation does not have any considerable influence on the growth of submerged sections in the absence of growth substances, a differential effect occurs in the presence of growth substances, the sections in the aerated solutions then showing increased elongation as compared to those in solutions with inadequate oxygen supply. As the authors state: "Oxygen becomes a limiting factor only when solutions which are not aerated are used in conjunction with factors favouring maximal growth, such as the addition of growth substances".

So here once more growth substance action is linked to oxygen consumption.

KELLEY (1947), investigating relationships between respiration and the uptake of water in the oat coleoptile, also provides evidence for the highly aerobic character of the growth, using the uptake of water as a measure. Several inhibitors known to inhibit carbohydrate metabolism in animal tissues also proved to inhibit the water uptake of coleoptile segments as well as the absorption of oxygen (respiration). Both processes were inhibited simultaneously and in the same concentration range. The stimulation of respiration and of water uptake by indole acetic acid is parallel to its stimulation of growth; uptake of water proved to take place under aerobic conditions only.

In tissue cultures of carrot and topinambour in media without sugar GORIS (1947 a) observed that absorption of water by the tissues was enhanced in the presence of indole acetic acid. In the same media the decrease of the sugar reserve in carrot tissue was found to be intensified by the addition of indole acetic acid (GORIS, 1947 b), particularly in the autumn, during which, probably in contrast to the situation in spring, the auxin content of the carrot tissue itself is low.

According to analyses by SUKHORUKOV AND SEMOVSKIKH (1946) the lower parts of normal coleoptiles contain more sugar than the higher ones. By application of growth substances these differences were found to become less pronounced, indicating that growth substances facilitate the diffusion of sugar in to the tissue.

In this connection it seems interesting to quote finally two papers concerning the action of 2,4-dichlorophenoxy-acetic acid on the germination of seeds and on the growth of different micro-organisms.

HSUEH AND LOU (1947) studied the germination of barley, a typical aerobic seed, and of rice, known to be able to germinate anaerobically. The germination of barley could be completely inhibited by a treatment with solutions of 2,4-D (e.g., 0.07%) whereas under the same conditions that of rice was only delayed. Similar effects were obtained by keeping the seeds under anaerobic conditions. So 2,4-D treatment seems to create a situation where oxygen is no longer available to the seeds. Also with other seeds this equivalence of anaerobic conditions and 2,4-D treatment could be established. Gas exchange analysis by means of the Warburg technique also indicated clearly that the seeds treated with 2,4-D cannot utilize very well oxygen in the air during germination and must find another source (e.g., fermentation) for energy supply. Rice possessing a highly functional fermentative mechanism meets the latter requirement and is therefore rather insensitive to 2,4-D, whereas the germination of barley, the seed of which lacks such a mechanism, is inhibited.

These investigations induced WORTH AND MCCABE (1948) to determine the effect of 2,4-D on aerobic, anaerobic and facultative anaerobic micro-organisms. Their results can be summarized as follows: Those organisms which require free oxygen for respiration

are—as the authors express it—“smothered” by 2,4-D; so they react in a manner similar to the barley seeds. If the organisms are capable of anaerobic respiration only, they are not affected by 2,4-D to any significant degree.

Though in both cases inhibitory actions mainly have been considered, the interesting results clearly have a bearing upon the problem under discussion, the more so as with very low concentrations of 2,4-D stimulating actions were observed with the seeds as well as with the micro-organisms.

All these data, to which still more could be added, indeed strongly suggest that in some way or other there exists a relation

growth substance action / a particular fraction of respiration (oxygen absorption, cf. aerobic character of growth) / water uptake / sugar transport / changes in the properties of the cell wall.

We are not yet informed, however, about the question whether all these functions are linked up in a series (in which case their exact sequence is not known either) or if they are partially linked up in a parallel sense. And so we do not know where the relation between the functions is of a primary or only of a secondary character.

To diagrammatize the situation still more roughly once more, we may state that for growth to occur

- A. nutritive matter and water has to be supplied to the cells, implying passage of membranes,
- B. inside the cell (cytoplasm) chemical processes under enzymatic regulation must proceed and products of these reactions have to go the way back—passing the barriers again—to be deposited in the walls, which
- C. in the meantime already must have been modified as to some of their properties.

In the beginning growth substance action has particularly been connected with C. Then one of us thought that A was the more important “site of action”, but now we know that both views have certainly been too one-sided and in our opinion there is sufficient evidence that the so-called primary reaction is “covered” by B. So growth substance action would rather be many-sided, being connected with B as well as with A and C. (As to the effects of growth substances on the membranes (A) the investigations of KONINGSBERGER *et al.* concerning the influence of growth substances on isolated protoplasts are of great importance (cf. KONINGSBERGER, 1947, 1948).

The results of our investigations described in this paper (cf. page 298) induce us to suppose that a rather considerable fraction of the growth substance (including the part “wasted” by aspecific adsorption) plays a rôle in A and that, properly speaking, for this function (concerning penetration) the highly active growth substances are not the most suitable compounds, but could better be replaced in a certain sense by the synergists described earlier in this paper. As to C, we must first of all await the results of the investigations referred to on page 300 in connection with the studies of BURSTRÖM.

The relatively small fraction acting in B most probably causes the kind of response which is the most specific for the highly active growth substances and it remains to be elucidated which is the exact function in the cytoplasm and what is the meaning of the particular spatial structure—reminding of that of a wetting agent or penetrant—which we found to be characteristic for the compounds with maximal activity.

As to the function in the cytoplasm, from the investigations discussed on page 301

it may be deduced with a high probability that enzymatic processes are involved (cf. also SWEENEY, THIMANN, 1942; WILDMAN, BONNER, 1946; BONNER, WILDMAN, 1946). As we had to reject the point of view that the auxins are part (prosthetic group) of an enzyme, or to express it in other words are acting *in* an enzyme, the remaining possibility is that they are acting *on* an enzyme system, *viz.*, are regulating its activity. This view (connected with the enzyme "activation" as expressed by AVERY *et al.*, and also considered by VAN OVERBEEK, 1947) is the more attractive as for some other hormones similar relationships particular enzymes have already been established. The work of CORI *et al.* (PRICE, CORI, COLOWICK, 1945; COLOWICK, CORI, STEIN, 1947) provided evidence that on the one side the adrenal cortical hormone and the diabetogenic hormone of the anterior pituitary gland and on the other side insulin are functioning (in opposite sense) as regulators of the activity of hexokinase, the enzyme which catalyses the phosphorylation of hexoses by ATP.

Some authors (cf. HARROW, 1947; MEYERHOF, 1948) are for this reason already inclined to generalize that hormones enhance or lower the activity of enzymes (containing *e.g.*, vitamins as prosthetic groups), in this way regulating the speed of turnover of the metabolites.

EYSTER (1943, 1946) already has put forward a view concerning the action of auxins, which in fact implies such a regulation of an enzyme system. The mechanism was understood as a release of certain enzymes, particularly of diastase, from a protein colloidal base, to which they were considered to be attached normally. This opinion was deduced from experiments of diastase on charcoal as a carrier and the influence of synthetic growth substances thereupon. For such important conclusions in a physiological sense this very simplified model system hardly seems to offer a sufficient base however. Moreover SMITH, LANGE LAND, AND STOTZ (1947), repeating EYSTER's experiments under more critically controlled conditions, did not obtain convincing evidence for a direct inhibition of free diastase by indole acetic acid nor for a significant influence on the absorption equilibrium of diastase on charcoal by the same acid.

In our opinion more promising starting points in this connection may be found in the work of AVERY *et al.* (cf. page 302) and a further analysis both qualitatively and quantitatively of the enzyme systems of *e.g.*, the *Avena* coleoptile and their influencing by growth substances *in vitro* and *in vivo* may procure basic information.

Following our trend of thought it must of course be our first aim to find connecting links which may lead to an understanding of the way in which a regulation of enzymatic activity by growth substances could occur and to a judgment of the question in how far the specific spatial structure of the highly active compounds is also of fundamental importance in this connection.

Now it might seem possible at first sight to start with ideas like those given by LASNITZKI (1947) in a survey on the relation between cell proliferation, carbohydrate breakdown and hydration of enzyme protein (for animal tissues), which considerations suggest "that the intensity of carbohydrate breakdown and consequently the rate of cell proliferation largely depends on the degree of hydration of corresponding enzyme proteins, so that, within limits, an increase in hydration stimulates and decrease inhibits that enzymatic activity". And one would ask if the uptake of water induced by auxin could be connected with similar relations in plant cells. (Cf. for the significance of swelling and shrinking of protoplasm, SEIFRIZ, 1946). Though it cannot wholly be excluded that such considerations may play some rôle in future discussions on auxin action, on second

thought objections arise which make it improbable that a solution of the problem will be found on this base.

First of all one has to take into consideration that a large part of the water taken up will be found back in the vacuole of the plant cells (absent in animal cells), whose volume is considerably enlarged during cell elongation and which does not contain proteins.

Moreover, recently LEVITT (1948), in a discussion on the uptake of water induced by auxin in aerated potato discs, pointed out on account of calculations that this uptake of water cannot, in a considerable measure, be due to protoplasmic protein hydration, and can only be explained by decreased wall pressure. Though it seems to us that with the latter statement the problem of water uptake has not yet been solved (if this were the only decisive factor, the osmotic pressure would have to decrease far more than it really does), it is clear that for our discussion other views are necessary than those indicated above.

Now it may be deemed possible that in the resting cells several enzymes are present in a bound (more or less inactive) form. Perhaps they are then bound to other cell constituents by complex relations (electrostatic forces). Or possibly they occur as an internal complex, this means that the "acting area" of the enzyme—not in the sense of prosthetic group, but in that of the atom grouping required for the catalytic actions—is masked as it were, *e.g.*, is located in the "interior" of the enzyme molecule. Disrupting of these complex relations then causes "liberation" of the active area and the enzyme action may start.

Now it is reasonable to suppose that anions with a special structure (like the growth substances) may influence similar complex relations in very low concentrations. This will occur if special relations exist between the structure of the acting anion and the structure of the enzyme to be activated or of the cell constituent to which the enzyme is bound.

Here we think also of the effect of detergents, particularly of those of anionic type, in relatively low concentrations on proteins, which implies a denaturation, *viz.*, liberation of -SH groups and increase in molecular asymmetry (*cf.* PUTNAM, 1948). Processes of this type, if occurring in a cell under influence of some agent may cause a definite physiological effect (*cf. e.g.*, the importance of a regulation of the effectiveness of -SH groups for the ratio cell division / cell elongation, NICKERSON, 1948). Taking into consideration that the (anionic) growth substances are related in principle to certain classes of detergents (wetting agents) as to their spatial structure, we are led to the question whether the auxins may be acting in a physico-chemically related sense on certain enzyme proteins, of course without thinking of a complete parallel of the denaturation caused by detergents.

We then would have to attribute to the auxins (and synthetic analogues with comparable action) a more or less specific affinity for enzyme systems which are important for cell elongation. Now indications for such a property have already been found by the investigations of AVERY *et al.* (*cf.* page 303). The fact that such low concentrations of growth substances are effective (of the order of  $10^{-18}$  g mol/cell, *cf.* WASSINK, 1946) could then also be understood.

Surveying the development of opinions concerning the action of auxin we believe that in this way we arrive at a situation where the efforts from biological and chemical side show a promising convergence.

The results of future investigations, to which we hope to contribute and of which the study of the interaction between growth substances and proteins (enzymes) will certainly constitute an important part, will decide in how far our suggestions have touched something essential concerning the action of growth substances.

#### SUMMARY

1. Continuing the investigations concerning the relation structure/activity of the plant growth substances, it is shown, that replacement of the lipophilic parts in the highly active compounds (derived from benzene, naphthalene or indole) by trichloro-acetyl-, tris-chloromethylacetyl-, di- or trialkylacetyl residues respectively, results in loss of activity. Only with high concentrations of the compounds, near the toxic level, weak curvatures are produced in the pea test, as already observed earlier for certain normal fatty acids. The acids with branched chains act more rapidly in this respect than their normal isomers. Similar relations are found with some structurally quite unrelated wetting agents, causing weak curvatures in the pea test particularly if the hydrophilic part is located in the centre of the lipophilic chain.

2. The importance of the spatial relation between the ring-system and the carboxyl group in the side chain, as derived formerly for the highly active compounds and reminding of the relations in the group of wetting agents and penetrants, is demonstrated once more for naphthalene-acrylic acid and derivatives. That the spatial structures ascribed to the compounds an account of their physiological activity are the correct ones, has in the meantime also been proved by means of their ultra violet absorption spectra (HAVINGA, NIVARD, 1948).

3. Comparison of the activities in series of compounds leads to the conclusion that for maximal activity, apart from the spatial relation between non-polar and polar parts of the molecule, a very definite balance between these lipophilic and hydrophilic parts (H.L. balance) is required. In this sense an upper limit is now also indicated for the requirement: "high interface activity of the non-polar part".

4. The effect of series of growth substances and related compounds on the tissue of the red beet (*Beta vulgaris rubra*)—by affecting the endo- and ectoplasmic membranes, the colouring matter leaves the vacuole and can be quantitatively measured—completely parallels that on the oleate coacervate (cf. preceding paper). So also with this biological object the effect, in a quantitative sense, is for the greater part the reverse of that which one would expect on account of the view that the primary growth substance action is mainly concerned with the protoplasmic membranes (influence upon the permeability).

5. If the primary reaction does indeed take place in the cytoplasm—an additional argument to those given in the preceding paper being the more rapid action observed for some neutral "precursors" of highly active acids as compared to that of these acids themselves—the result of the beet test can be explained in a plausible way. Increase of the lipophilic character of a compound beyond a certain limit may namely cause too strong an interaction with (adsorption to) the membranes, preventing the attainment of the "cytoplasmic" concentration required for maximal activity (apart from a direct effect of changed membrane properties).

6. It is deduced that a properly composed mixture of "underdosed" highly active growth substance and a supplementary quantity of an acid, weakly active or inactive for the reasons indicated under 5, should equal the maximal effect of the growth substance alone, even if the total number of molecules is less. Such synergistic effects of certain types of acids could indeed be established in the pea test. From the proportions found in this respect it is concluded that only a small fraction of a growth substance in a biological system is actually required for the primary reaction and that the greater part is adsorbed to the membranes (considered to perform a physiological function), has some action on the cell wall and is possibly adsorbed aspecifically at other places ("waste").

7. These findings are discussed in relation to views already put forward earlier by F.W. WENT (1939, preparatory reaction — primary reaction) and to the evidence for a two-phase growth reaction, provided by the investigations of BURSTRÖM (1941–1942–1945).

8. A short review is given of the most important biological investigations concerning growth substance action. The data suggest that a relation exists: growth substance action/a particular fraction of respiration (oxygen absorption, cf. aerobic character of growth)/water uptake/sugar transport/changes in the properties of the cell wall.

As to the function of the growth substances in the cytoplasm it is highly probable that enzymatic processes are involved.

9. Arguments are given which lead to rejection of the view that the auxins are part (prosthetic group) of an enzyme, in other words, in our opinion the auxins do not act catalytically as co-enzymes. The remaining possibility then would be that they act on an enzyme system, viz., that they function

as regulators of enzymatic activity. Suggestions are given concerning the way in which this may occur, taking into consideration the results of the chemical investigations. This implies that in future investigations the interaction between growth substances and proteins (enzymes) will particularly have to be studied.

## RÉSUMÉ

1. En poursuivant les recherches sur la relation structure/activité des substances de croissance, on a montré que le remplacement des parties lipophiles dans les composés hautement actifs (dérivés du benzène, du naphthalène, ou de l'indol) respectivement par des résidus trichloroacétyl, tris-chlorométhylacétyl, di- ou trialkylacétyl, entraîne une perte d'activité. C'est seulement avec de fortes concentrations de ces composés, voisines de la dose toxique, que l'on obtient de faibles courbures dans le test de *Pisum*, comme cela a déjà été montré pour certains acides gras normaux. Dans ce domaine, les acides possédant des chaînes ramifiées, agissent plus rapidement que leurs isomères normaux. Des relations semblables ont été trouvées chez certains agents mouillants de structure tout-à-fait différente, qui entraînent de faibles courbures dans le test de *Pisum*; particulièrement si la partie hydrophile est localisée au centre de la chaîne lipophile.

2. Nous démontrons à nouveau pour l'acide naphthalène-acrylique et ses dérivés l'importance de la relation spatiale entre le système cyclique et le groupe carboxyle de la chaîne latérale, trouvée d'abord pour les composés hautement actifs et rappelant les relations du groupe des agents mouillants et pénétrants. L'étude des spectres d'absorption dans l'ultraviolet a montré, en même temps, que les structures spatiales attribuées aux composés d'après leur activité physiologique, étaient correctes (HAVINGA, NIVARD, 1948).

3. La comparaison des activités d'une série de composés conduit à la conclusion suivante: pour l'activité maxima, à part les relations spatiales entre les parties non-polaires et polaires de la molécule, il est nécessaire qu'il existe un équilibre bien défini entre les parties hydrophiles et lipophiles (balance H.L.). Maintenant, dans ce sens, une limite supérieure est indiquée aussi pour la condition requise: "haute activité interfaciale de la partie non-polaire".

4. L'effet de séries de substances de croissance et de composés voisins sur le tissu de la betterave rouge (*Beta vulgaris rubra*) — en agissant sur les membranes endoplasmique et ectoplasmique, la substance colorante quitte les vacuoles et peut être mesurée quantitativement — est tout à fait parallèle à celui exercé sur un coacervat d'oléate (voir publication précédente). Ainsi, avec cet objet biologique, l'effet, dans un sens quantitatif, est en grande partie l'inverse de celui qu'on pourrait attendre d'après l'idée que l'activité primaire de la substance de croissance est nettement liée aux membranes protoplasmiques (influence sur la perméabilité).

5. Si la réaction primaire se produit dans le cytoplasme — un argument qui s'ajoute à ceux donnés dans la publication précédente étant l'action beaucoup plus rapide de certains "précurseurs" neutres des acides hautement actifs, en comparaison avec l'action de ces acides eux-mêmes — le résultat du test de la betterave peut être expliqué d'une manière plausible. L'augmentation du caractère lipophile d'un composé au delà d'une certaine limite, doit entraîner une interaction trop grande avec les membranes, ce qui empêche l'obtention de la concentration, dans le cytoplasme, nécessaire pour l'activité maxima.

6. On en déduit qu'un mélange convenable d'une substance de croissance hautement active, mais à dose trop faible, avec une quantité supplémentaire d'un acide, faiblement actif ou inactif pour les raisons indiquées au paragraphe 5, peut égaler l'effet maximum de la substance de croissance seule, même si le nombre total de molécules est inférieur. De tels effets synergiques de certains types d'acides ont été établis dans le test de *Pisum*. D'après les proportions trouvées dans cette étude, on conclut que seule une petite fraction de la substance de croissance, dans un système biologique, est nécessaire pour la réaction primaire, la plus grande partie de la substance étant adsorbée sur les membranes (fonction physiologique aussi) et exerçant quelque action sur la paroi de la cellule.

7. Ces résultats sont discutés relativement aux idées émises par F. W. WENT (1939, réaction préparatoire, réaction primaire) et aux arguments en faveur d'une réaction de croissance en 2 phases selon les recherches de BURSTRÖM (1941, 1942, 1945).

8. On donne une brève revue des recherches biologiques les plus importantes concernant l'action des substances de croissance; les résultats suggèrent qu'il existe une relation entre l'action d'une substance de croissance / une part déterminée de la respiration (absorption d'oxygène, voir le caractère aérobie de la croissance) / l'absorption d'eau / le transport de sucre / des modifications des propriétés des parois de la cellule. Quant à la fonction des substances de croissance dans le cytoplasme, il est fort probable que des processus enzymatiques s'y trouvent engagés.

9. D'après les arguments donnés, nous sommes amenés à rejeter l'idée que les auxines font partie (comme groupement prosthétique) d'une enzyme. En d'autres termes, dans notre opinion, les auxines n'agissent pas catalytiquement comme co-enzymes. La possibilité qui subsiste alors est la suivante: Elles agissent sur les systèmes enzymatiques, c'est à dire qu'elles fonctionnent comme régulateurs



de l'activité enzymatique. Certaines suggestions sont faites sur le mode d'action, et ce, d'après des résultats de recherches chimiques. Ceci indique que des recherches ultérieures sur l'action mutuelle des substances de croissance et des protéines (enzymes) seraient particulièrement indiquées.

### ZUSAMMENFASSUNG

1. In Fortführung der Untersuchungen über das Verhältnis zwischen der Struktur und der Aktivität der Wuchsstoffe wurde gezeigt, dass der Ersatz der lipophilen Molekülteile in den hochaktiven Substanzen (welche vom Benzol, Naphthalin oder Indol abgeleitet sind) durch Trichloracetyl-, Tris-chloromethylacetyl-, Di- oder Trialkylacetylreste einen Aktivitätsverlust mit sich bringt. Nur bei hohen, nahe der Giftigkeitsgrenze gelegenen Konzentrationen dieser Verbindungen konnten im Erbsentest schwache Krümmungen hervorgerufen werden, wie wir sie schon früher bei Anwendung einiger normaler Fettsäuren beobachtet hatten. Die Säuren mit verzweigter Kette wirken hier rascher als ihre normalen Isomeren. Ähnliche Zusammenhänge wurden bei einigen, strukturell gar nicht verwandten Netzmitteln beobachtet, die im Erbsentest schwache Krümmungen hervorrufen, insbesondere wenn der hydrophile Teil in der Mitte der lipophilen Kette gelegen ist.

2. Die Bedeutung der räumlichen Abhängigkeit zwischen dem Ringsystem und der in der Seitenkette gelegenen Carboxylgruppe, die schon früher für die hochaktiven Substanzen abgeleitet wurde und an die Verhältnisse in der Gruppe der Netzmittel erinnert, wurde erneut für die Naphthalenacrylsäure und ihre Derivate bewiesen. Die Richtigkeit der diesen Verbindungen auf Grund ihrer physiologischen Aktivität zugeschriebenen räumlichen Anordnung wurde inzwischen auch mit Hilfe ihrer Ultraviolettabsorptionsspektren bestätigt (HAVINGA, NIVARD, 1948).

3. Ein Vergleich der Aktivitäten in einer Reihe von Verbindungen führt zu der Schlussfolgerung, dass abgesehen von dem räumlichen Verhältnis zwischen polaren und nicht polaren Molekülteilen auch noch ein bestimmtes Gleichgewicht zwischen diesen hydrophilen und lipophilen Teilen (H.L.-Balanç) herrschen muss damit die Aktivität maximal sei. In diesem Sinne wird eine nun auch obere Grenze angedeutet für die Bedingung: "hohe Grenzflächenaktivität der nicht polaren Teile".

4. Die Wirkung einer Reihe von Wuchsstoffen und verwandter Verbindungen auf das Gewebe der roten Rübe (*Beta vulgaris rubra*) verläuft parallel mit der für ein Oleatkoazervat beobachteten (siehe vorhergehende Mitteilung)—durch eine Veränderung in der Membrane des Endo- und des Ectoplasmas verlässt der Farbstoff die Vakuole und kann quantitativ bestimmt werden. So ist also auch an diesem biologischen Objekt die Wirkung im quantitativen Sinne zum grössten Teil den Erwartungen entgegengesetzt wenn man sich auf die Annahme stützt, dass die primäre Wuchsstoffwirkung hauptsächlich die Protoplasmamembranen betrifft. (Beeinflussung der Permeabilität).

5. Findet die primäre Reaktion wirklich im Cytoplasma statt—ein weiteres Argument zu den in der vorhergehenden Mitteilung angeführten ist die rascherer Wirkung von einigen "Vorstufen" hochaktiver Säuren im Vergleich zur Wirkung dieser Säuren selbst — dann können die Ergebnisse des Rübens tests verständlich gemacht werden. Überschreitet nämlich der lipophile Charakter einer Substanz einen gewissen Grenzwert, dann kann durch zu starke Wirkung auf die Membrane (Absorption), die, für eine maximale Aktivität nötige Konzentration im Protoplasma nicht mehr erreicht werden (dies ganz abgesehen von der direkten Auswirkung der veränderten Eigenschaften der Membrane).

6. Hieraus wird abgeleitet, dass die Wirkung eines "unterdosierten" Wuchsstoffes zusammen mit einer Säure, die aus den unter 5. angeführten Gründen inaktiv oder wenig aktiv ist, der maximalen Wirkung des allein zugeführten Wuchsstoffes gleich sein sollte, auch wenn die Gesamtzahl der Moleküle kleiner ist.

Solche synergetische Effekte gewisser Säuretypen konnten in der Tat mit dem Erbsentest festgestellt werden. Aus den hier gefundenen Proportionen schliessen wir, dass in einem biologischen System nur ein kleiner Teil der Wuchsstoffmenge für die primäre Reaktion verbraucht wird, während der grössere Teil an den Membranen absorbiert wird (physiologische Funktion), auf die Zellwand wirkt und möglicherweise unspezifisch an anderen Stellen absorbiert wird (Verlust).

7. Diese Ergebnisse werden in Verbindung mit den früher von F. W. WENT geäusserten Anschauungen (1939 "vorbereitende" Reaktion — primäre Reaktion) und den von BURSTRÖM (1941, 1942, 1945) gelieferten Beweisen für eine Wachstumsreaktion in zwei Fasen diskutiert.

8. Wir geben eine kurze Übersicht über die wichtigsten biologischen Untersuchungen die die Wirkung der Wuchsstoffe betreffen. Die Ergebnisse lassen zwischen den folgenden Faktoren einen Zusammenhang vermuten: Wuchsstoffwirkung / ein bestimmter Teil der Atmung (Sauerstoffaufnahme, man denke an den aeroben Charakter des Wachstums) / Wasseraufnahme / Zuckertransport / Veränderungen in den Eigenschaften der Zellwand. Was die Wirkung der Wuchsstoffe im Protoplasma betrifft so handelt es sich hier höchstwahrscheinlich um enzymatische Vorgänge.

9. Auf Grund der angeführten Argumente sollte die Ansicht verworfen werden, dass die Auxine (als prosthetische Gruppe) einen Teil eines Enzyms bilden; unserer Ansicht nach wirken die Auxine also nicht katalytisch als Coenzyme. Es bleibt dann die Möglichkeit einer Wirkung auf ein Enzym

als Regulator der enzymatischen Aktivität. Auf Grund der chemischen Untersuchungen schlagen wir einige mögliche Mechanismen für diese Wirkung vor. In Zukunft wird daher die gegenseitige Wirkung von Wuchsstoffen und Proteinen (Enzymen) besonders untersucht werden müssen.

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## STUDIES OF THE REACTION BETWEEN PROTEINS AND REDUCING SUGARS IN THE "DRY" STATE

### I. THE EFFECT OF ACTIVITY OF WATER, OF $p_H$ AND OF TEMPERATURE ON THE PRIMARY REACTION BETWEEN CASEIN AND GLUCOSE

by

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While it is now believed that products of a reaction between the free amino groups of amino acids or proteins and reducing sugars may contribute to the desirable colour, aroma and flavour of certain processed foods, they are apparently concerned also in the deterioration of a number of other food products and the reaction has obvious possibilities of interest in non-food systems containing protein or protein fragments and carbohydrate.

In recent work on the deterioration of skim milk powder HENRY, KON, LEA, AND WHITE<sup>1</sup> obtained clear indications of a reaction between the free amino groups of the milk protein and lactose leading to undesirable physical and chemical changes in the powder and to a reduced availability to young rats of the lysine present. The use of a simpler system than milk powder, however, was essential for further elucidation of the changes involved and a general investigation has therefore been undertaken of the interaction between proteins and reducing sugars. Casein was chosen as starting material for the first experiments, despite its known lack of homogeneity, because it accounts for over 80% of the protein of milk, and because the quantities of material required precluded the use of crystalline  $\beta$ -lactoglobulin. This latter protein is present as only a very minor constituent of milk protein, and is now itself believed to be heterogeneous.

Practically nothing appears to have been published on the reaction between protein and sugar at low activities of water, and very little on the reaction in solution. SHIGA<sup>2</sup>, in a single experiment with egg albumin and glucose in solution (100 moles glucose per free amino group) observed only a comparatively slight reaction which increased with increasing  $p_H$  over the range  $p_H$  7-9. Several earlier workers had also obtained evidence by various methods of a slight combination between glucose or fructose and proteins in aqueous solution at  $p_H$  values more alkaline than 7. PRZYLECKI AND CICHOCKA<sup>3</sup> investigated the formation of "covalence-like symplexes" from carbohydrates and proteins in aqueous solution by allowing protein solutions of 5-7% concentration to react with saturated or half saturated sugar solutions at 5-12° C and at a  $p_H$  initially of 7-9 and finally of 9-10 for 2-4 days. A marked proportionality was reported between the

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lysine content of the protein used and the amount of sugar found in the product after precipitation with alcohol. The combination however appeared to be unstable and was split at physiological  $p_H$ s.

A considerable amount of information is available concerning the reaction between amino acids or simple peptides and sugars in solution, but is often conflicting. Evidence for combination has been obtained by cryoscopic, optical rotation and electrometric titration methods, as well as by the loss of VAN SLYKE nitrogen and by the increase of reducing power towards methylene blue or 2:6 dichlorophenolindophenol. The reaction is slow and does not approach completion even after many hours at laboratory temperature. At high concentrations and temperatures extensive secondary reactions occur leading to the production of complex, highly coloured substances of high molecular weight ("melanoidins"), and eventually to charring and the copious evolution of gas.

Evidence as to the effect of  $p_H$  on the rate of reaction has been conflicting. Some of the earlier data show a marked optimum in the region of  $p_H$  7-8<sup>4, 5</sup>, while others indicate a continued increase in the rate of reaction with increasing alkalinity<sup>6, 7</sup>. SHIGA<sup>2</sup> concluded that the amount of combination of amino acid with glucose increases with the  $p_H$  value, while that of di- or tripeptides with glucose has an optimum at  $p_H$  7 or 8. FRANKEL AND KATCHALSKY<sup>8</sup> found that the percentage combination at equilibrium of glucose with glycine ranged from zero at  $p_H$  6 to over 80% at  $p_H$  10 and above, while for leucylglycine the corresponding  $p_H$  values were 7 and 11.5, no  $p_H$  optimum being observed in either case.

Free amino groups are not the only reactive centres in the protein molecule which might combine with aldehyde groups. With cysteine in aqueous solution, for example, the SH as well as the  $NH_2$  group has been shown to react with glucose to give a fairly stable thiazole ring compound which has been isolated<sup>9, 10</sup>.

## METHODS

### *Sodium caseinate*

Casein was prepared from fresh, unheated cow's milk, after centrifugal separation of the cream, by the method of COHN AND HENDRY<sup>11</sup>, which avoids exposure to organic solvents or to alkaline conditions. After precipitation at  $p_H$  4.6 and washing, the isoelectric casein was dispersed again by the slow addition, with stirring, of sodium hydroxide to  $p_H$  6.3, and the "solution" carefully dried in the frozen state under high vacuum. The product, after removal from the drying trays was held overnight in an atmosphere at 60% R.H., broken down and mixed by very brief agitation with the stainless steel blades of a WARING blender, and stored in sealed containers at -20° C until required. The sodium caseinate thus prepared consisted of light white glistening flakes which were readily dispersible in water.

### *Casein-glucose mixtures*

For the preparation of 'dry' casein-sugar reaction mixtures part of the stock sodium caseinate was dispersed in water to a 2% solution, glucose was added in quantity exactly equivalent to the determined free amino N content of the protein (ca 11% by weight of the anhydrous protein) and the liquid, after cooling to 0° C, was shock frozen by rapid evaporation in the freeze-drier. In some cases the  $p_H$  of the liquid was adjusted to some desired figure by the addition of HCl or NaOH before drying. After freeze-drying the samples were held at 0° C in an atmosphere at 56% R.H. (corresponding to approximately 60% R.H. at 20° C) for 2 days to reduce moisture changes during subsequent handling, and mixed in the WARING blender before use. Owing to the relatively enormous surface area exposed by these products they could readily be equilibrated with any desired atmosphere.

### *Equilibration to known water-vapour pressures*

Since it was considered likely that the influence of water on chemical reactivity within the system would be connected more closely with the activity of water as indicated by vapour pressure measurements than with total water content as determined by some arbitrarily chosen heating or drying method, storage conditions have been defined, wherever possible, in terms of the vapour pressure of

the material expressed as percentage relative humidity. Since, moreover, the rate of transference of moisture from environment to sample or vice-versa has a much smaller temperature coefficient than have the chemical reactions under investigation the sample was usually equilibrated over dilute sulphuric acid solution to the required moisture content at a low temperature prior to storage at a higher temperature.

The pressure of water-vapour over solutions of sulphuric acid of known concentration changes regularly with the temperature, and the magnitude of the effect is comparatively small. Proteins, however, show so marked a change in capacity for 'binding' water with change in temperature that some preliminary investigation with the experimental material to be used was necessary.

#### *The water-relations of the casein-glucose system*

Investigation of the water relations of the casein-glucose system was complicated by the necessity for equilibrating the samples sufficiently rapidly to avoid changes resulting from commencement of the amino-aldehyde and its secondary reactions. This meant the use of small weights and large surface areas, which militated against the attainment of a high degree of accuracy. The data given in Fig. 1 were obtained by bringing the weighed samples of casein-glucose into equilibrium first with an atmosphere of 85 % R.H. and subsequently with the required relative humidity at 10° C. During the 2 or 3 days required for this process chemical change was negligible. The samples were then transferred to atmospheres at 37° C so chosen that only a very small further quantity of water would be given up, and weighed *in situ* at intervals up to 48 hours. From the curves obtained by plotting change in weight against time a correction for any loss of volatile matter due to commencement of the chemical reaction could be made. In general, the correction was zero or very small for the driest samples, but was quite appreciable in the region of maximum reaction velocity (ca 70 % R.H.). The moisture contents of the various samples were then obtained by relating them to the original material, the water content of which was found by drying *in vacuo* over anhydrous (magnesium perchlorate) at 0° C for 3 weeks and then at 37° C to constant weight.

At temperatures above 37° C the rate of chemical change was so great that adsorption data could not be obtained directly. The isotherms required at 55, 70 and 90° C were therefore derived with sufficient accuracy from those at 10 and 37° C by the extrapolation of isosteres on a logarithmic plot<sup>13</sup>. With the aid of the appropriate isotherms samples of casein-glucose could be adjusted at 10° C to water contents which would be in equilibrium with a wide range of atmospheres at higher temperatures, and the initial stages of the reactions would not therefore be complicated by unnecessary gain or loss of water.

#### *Storage*

a) *Constant relative humidity.* Many of the experiments were carried out in a room with rapid air circulation, thermostatically controlled at  $37 \pm 0.1^\circ \text{C}$ . Six or eight 200 mg samples in thin-walled glass tubes, after equilibration at 10° C were stoppered, heated rapidly to 37° C, opened and transferred to airtight jars containing sulphuric acid producing the required relative humidity. A similar technique was employed for storage at 28.5, 20, 10 and 0° C.

The experiments at 55° C/70 % R.H. and 70° C/70 % R.H. were carried out in an airtight jar partly filled with sodium nitrate solution of suitable strength, and maintained at reaction temperature by total immersion in a water thermostat. The samples, after adjustment to the correct moisture content at 10° C were compressed against the flat bottoms of the tubes to improve thermal contact with the glass, and rapidly heated by partially immersing the tubes in the sodium nitrate solution. A thin film of Silicone grease on the outsides of the tubes prevented 'creeping' of the salt solution into the contents.

b) *Constant moisture content.* Samples of 100 mg each, with moisture contents adjusted to 6.0, 10.3 and 13.9 %, were heated in closed containers in which the free space was so small that it could be saturated at reaction temperature by the evaporation of less than 2 % of the water present in the material. At 37° C the material was packed into glass specimen tubes of volume approximately 2 ml, closed with rubber stoppers and sealed with wax; at 70 and 90° C it was compressed into discs approximately 2 cm in diameter and 0.5 mm thick between two sheets of pure tin foil which were then sealed by a triple fold along the open edges. This latter type of package was used because it not only had a small internal volume but could also be heated rapidly between two heavy copper blocks (ca 3 kg each) contained in an electric oven. Both blocks were drilled for thermometers and the upper carried an insulated handle to facilitate rapid movement. After heating for the desired period the

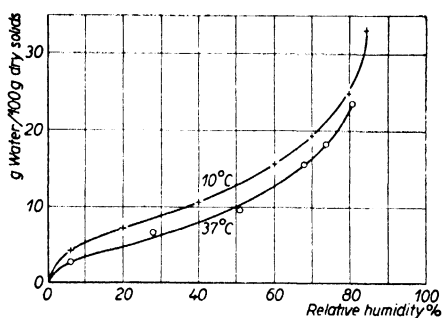


Fig. 1. The relation between water content and equilibrium relative humidity for the casein-glucose system at pH 6.3

foil-wrapped sample was removed, chilled between cold metal blocks and weighed (for estimation of loss of moisture) prior to chemical examination. This technique is an adaptation of that employed by WRIGHT<sup>13</sup> in an investigation of the effect of heat on the solubility of milk powder.

#### *Estimation of free amino groups*

The determination of free amino-N was carried out by the manometric method of VAN SLYKE according to the procedure previously described<sup>14</sup>, employing a reaction time of 30 minutes at 20° C and a correction of +2 mg amino-N/g total N to allow for completion of the reaction. The dry sample was usually dispersed by 'wetting' with 1 ml of glacial acetic acid in the graduated cup of the apparatus, followed immediately by dilution with water and washing into the reaction chamber. There was no evidence that this procedure caused any liberation of combined amino groups. Alternatively, the sample was dispersed in water by standing for three hours in a 5 ml cup fitted with a tap, through which the viscous solution could be drawn into the apparatus. This method was used, after a preliminary shredding with scissors, for the thin discs of compressed material produced by the copper block technique.

#### *Measurement of colour*

The stored samples were packed in a standardized manner into small porcelain dishes and examined in the LOVIBOND-SCHOFIELD Tintometer. Illumination was by C.I.E. Standard Illuminant B, consisting of gas-filled metal filament lamps operating at a colour temperature of 2848° K, used in combination with the specified colour filter solutions. Little use of the obturator vane was necessary over the limited range of brightness encountered in the experiments and, for simplicity, colours have been recorded as the sum of the yellow and red units used.

## RESULTS

### CHANGES IN FREE AMINO-N

#### *Effect of activity of water on reaction velocity*

The results summarized in Figs 2A and 2B were obtained in two experiments covering respectively 0.64 and 0.2 days at 37° C. In the first, casein-glucose weighed at 60% R.H. was adjusted to the required moisture contents and relative humidities without any pre-treatment. Some of the samples therefore reached equilibrium by

hydration, and some by dehydration.

It is known that a number of proteins display a slight hysteresis between 15 and 65%<sup>15</sup>, material equilibrated by dehydration retaining appreciably more moisture than similar material equilibrated to the same relative humidity by hydration. In the second experiment, therefore, the casein-glucose was roughly equilibrated at 13-15% R.H. before adjustment to the required value, so that all the samples attained equilibrium by absorption of water; the data for 1 and 2 days in Fig. 2B appertain to this experiment. No major discrepancy between the rates of reaction in the two series of samples is apparent.

While this work was in progress, the data of MELLON, KORN, AND

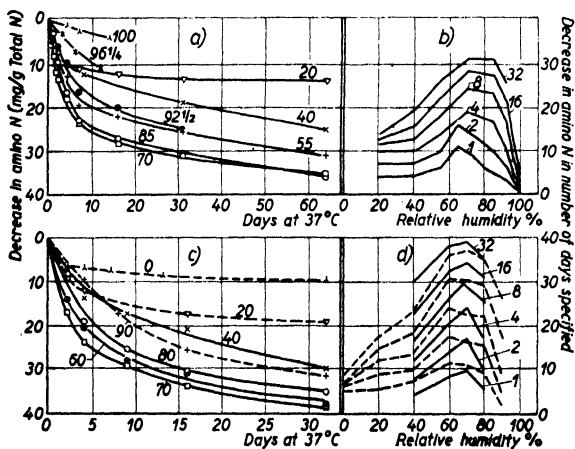


Fig. 2. The relation between activity of water and loss of free amino-N in the casein-glucose system at 37° C and pH 6.3. A and B were obtained without pre-adjustment of water content, C and D by dehydration (continuous line) or hydration (broken line).

HOOVER<sup>16</sup> appeared, according to which the hysteresis water of isoelectric casein, which can amount to as much as 2% at 51% R.H., is still present to the extent of approximately

1.2% at 6% R.H. and requires virtually complete drying for its removal. A double range of samples was therefore prepared from a fresh preparation of sodium caseinate for a third experiment. For the dehydration series, the weighed samples of casein-glucose were equilibrated at 10° C and 85% R.H. before adjustment to the correct moisture contents and transference to 37° C for storage at the required relative humidities. For the hydration series, the casein-glucose was dried as intensively as was practicable without inducing chemical change (anhydrous in vacuo for several weeks at 0° C followed by 4 days at 20° C) before adjustment of the moisture contents at 10° C and storage at 37° C.

Changes in free amino-N during storage of the dehydration and of some of the hydration materials are shown in Fig. 2C; the remaining hydration curves are omitted for the sake of clarity. While the behaviour of dehydration and hydration samples on storage was not identical (Fig. 2D), the differences were considered to be of doubtful significance and it was concluded that the rate of disappearance of the protein amino groups was mainly determined by the equilibrium relative humidity of the system and was not seriously influenced by the route by which equilibrium had been attained. Measurement of the weight of the samples at intervals during storage failed to indicate any considerable difference between the water contents of the corresponding hydration and dehydration materials. It must be borne in mind, however, that any conversion of glucose from the hygroscopic supercooled "glass" to the comparatively non-hygroscopic crystalline form, which might occur as a result of exposure to a moist atmosphere, would tend to counterbalance the effect of hysteresis in the protein.

To obtain 0% R.H. the casein-glucose was dried over anhydrous at 0° C for several weeks before storage over anhydrous at 37° C. A sample dried for a shorter period and stored over 'concentrated' sulphuric acid, which also should provide an atmosphere of virtually 0% R.H., showed an appreciably higher rate of reaction, thereby emphasizing the difficulty of removing chemically active water from the nearly dry protein. Toluene and chloroform were present in the atmospheres of the vessels containing the 100, 96 ¼, 92 ½ and 90% R.H. samples, in order to retard microbial attack. In separate runs at 85% R.H. with and without the antiseptic no difference in reaction rate attributable to its presence could be detected. The '100%' R.H. sample, which was stored over water, was still absorbing water at the end of the experiment. The low reaction rate observed for this material (Figs 2A and 2B) must, therefore, have been too high. A sample stored in concentrated aqueous solution in the presence of toluene showed a very slow and approximately linear *increase* in free amino N, presumably as the result of hydrolysis.

The results at 92 ½ % R.H. and below disclose a relatively rapid initial drop in amino-N, varying greatly in rate and extent with relative humidity, and falling away after 5-15 days to a slow drift downwards. The data at 96 ¼ and 100% could not be completed because of the appearance of signs of microbial attack, but showed a much slower initial rate of reaction. Figs 2B and 2D, in which the reduction in free amino N after various periods of storage has been plotted against the relative humidity of the atmosphere in equilibrium with the sample, show that the initial rate of combination of the free amino groups of casein with glucose displays a comparatively sharp maximum in the region of 65 or 70% R.H., and falls away at higher and lower humidities. At low moisture contents the reaction slows down or stops after only a small proportion of the free amino groups have reacted.



*Effect of  $p_H$* 

Portions of the stock sodium caseinate ( $p_H$  6.3), after dispersal in water and the addition of one equivalent of glucose, were adjusted to  $p_H$  values in the regions of 2, 3, 4.6, 7, 8, 9 and 10, shock frozen, freeze dried, equilibrated at 0 or 10° C, and stored at 37° C and 55 or 70% R.H. Sodium hydroxide solutions were used for controlling the vapour pressures of the more alkaline samples. Control samples of protein alone at  $p_H$  3, 9 and 10, without glucose, remained unchanged in free amino group content during storage for over 2 months at 37° C and 70% R.H. A decided tendency was noticed, however, for the amino-N content of the most acid samples, before storage, to be higher than the normal initial value of 53–55, and the rate of combination of protein and sugar at  $p_H$  2 could not be determined with any accuracy, since at this acidity the apparent free amino content of a casein-glucose mixture (as well as of casein alone) *increased* during storage at 37° C and 55% R.H., possibly as a result of the conversion of amide groups to ammonia which is known to interfere with the VAN SLYKE deter-

TABLE I  
THE EFFECT OF  $p_H$  ON THE RATE OF LOSS OF FREE  
AMINO-N IN CASEIN-GLUCOSE AT 37° C AND 55 OR  
70% R.H.

$p_H$	Initial rate of loss of amino-N*	
	55% R.H.	70% R.H.
3.0	4	7
4.6	—	14
6.3	35	44
7.0	41	68
8.0	55	71
9.0	53	—
10.0	63	—

\* Reciprocal of the number of days required for reaction of 20% of the free amino-N  $\times$  100

mination<sup>17</sup>. Correction for the 'blank' determination without glucose indicated a rate of reaction at  $p_H$  2 even slower than the already slow rate obtaining at  $p_H$  3 and 55% R.H. Measurement of the rate of reaction between casein and glucose at  $p_H$  4.6 and 70% R.H., was also unsatisfactory owing to the impossibility of making an intimate homogeneous mixture as casein is virtually insoluble at this  $p_H$ , and the value obtained may be low.

Table I shows that the initial reaction between casein and glucose is comparatively slow under acid conditions, and increases with increasing  $p_H$  at least as far as  $p_H$  8. The course of the reaction beyond this point was difficult to ascertain by the technique employed. Storage at 55% R.H. for example showed, in one experiment, a much slower rate of increase (Table I), and in another even a slight falling off in reaction rate between  $p_H$  8 and 10. The freshly prepared materials at  $p_H$  9 and 10, however, possessed appreciably lower initial amino-N values than did the samples at  $p_H$  6.3, 7 and 8, and if the experimental data were corrected on the assumption that the difference in initial values (2½ and 4 units in the case of the  $p_H$  10 samples) represented reaction of very labile groups during preparation then a comparatively smooth extension of the  $p_H$  3–8 curve was obtained.

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At 70% R.H. much larger decreases in reaction rate above  $p_H$  8 were observed and when combination became very slow after about 30 days at 37° C the amino-N content of the  $p_H$  9 and  $p_H$  10 samples had only fallen by some 26 and 19 units respectively, in place of the 34 units observed at  $p_H$  6.3, 7 and 8. Considerable losses of free amino-N observed in samples at  $p_H$  9 and 10 during preparation and equilibration prior to storage were again suggestive of a very rapid reaction at alkaline  $p_H$  of some of the free amino groups present.

It seems reasonable to conclude therefore, on the evidence available, that the rate of combination of the free amino groups of casein with glucose probably continues to increase with increasing alkalinity beyond  $p_H$  8, although the methods used were unable to establish the point with certainty.

#### *Effect of temperature*

Casein-glucose, pre-dried to 13% R.H. for four days at 0° C before adjustment at 10° C to the required moisture content, was held in equilibrium with an atmosphere of 70% R.H. at temperatures of 0, 10, 20, 28.5, 37 and 55° C, as described above, and examined at intervals for free amino-N content and for colour. Above 55° C the reaction became so rapid that appreciable errors could be introduced during the brief time required for the samples to attain reaction temperature and to adsorb or desorb any small quantities of water necessitated by slight errors in the isotherms. A further experiment was, however, performed at 70° C to obtain a figure of less accuracy.

When the results were plotted it was found that all the curves of amino loss against time were of the same general shape and tended to the same final value. The rates could therefore be compared quite adequately by considering only the initial reaction rate determined by drawing the tangent at zero time to the smoothed curve. A number of readings were taken at short reaction times to ensure the accuracy of the initial portion of the curve. The values for the initial rate, expressed as % loss of amino-N per hour, were 0° C-0.0015; 10° C-0.0067; 20° C-0.034; 28.5° C-0.21; 37° C-0.71; 55° C-9.3; 70° C-58.

A second series of samples was heated at constant moisture content, partly for comparison with the constant relative humidity results and partly because this system was the only practicable one at the higher reaction temperatures. The casein-glucose was first dried over magnesium perchlorate at 10° C, equilibrated at 10° C to three different moisture contents of 6.0, 10.3 and 13.9% and then heated at 37° C in glass tubes or at 70 or 90° C between copper blocks as previously described. The accuracy of the method at high temperatures was limited by the fact that the tinfoil packets allowed small losses of moisture over long periods, and by unavoidable errors of the order of  $\pm 5$  seconds in the time and  $\pm 0.2^\circ$  C in the temperature of heating. Each reading, however, was taken in triplicate, and the general agreement was found to be good. Smooth curves were plotted and the initial rates determined as before. Casein heated alone at 90° C for one hour under the conditions of the experiment showed no significant loss of amino-N.

The results of the two experiments are summarized in the ARRHENIUS plots of Figs 3A and 3B, and the following conclusions can be drawn.

a) The data at constant relative humidity show a good straight line relationship, with a temperature coefficient of 5.4 between 15 and 25° C, or 3.6 between 60 and 70° C. A value of 29000 cal for the apparent energy of activation can be deduced from the slope of the graph.

*References p. 324/325.*

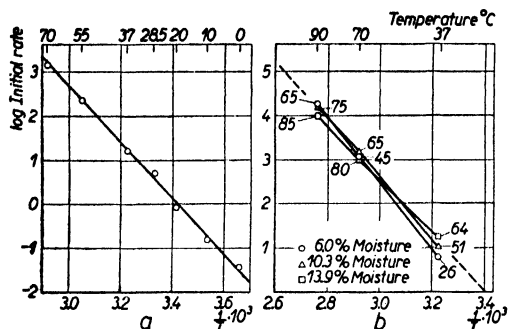


Fig. 3. The relation between temperature and the initial rate of loss of free amino-N in casein-glucose (A) at a constant relative humidity of 70% and (B) at constant water contents of 6.0, 10.3 and 13.9%. (Rate is measured in % loss of free amino-N per day. Figures on graph (B) are the approximate relative humidities at the specified temperatures).

c) The reaction rates at 70 and 90° C are sufficiently consistent with those observed at lower temperatures to indicate that the rate increases uniformly with temperature from 0 to 90° C.

b) Whereas at 37° C the sample containing 13.9% of moisture reacted fastest, and that containing 6.0% was slowest, the opposite applied at 90° C. At 70° C the 10.3% sample was faster than the other two. These apparent discrepancies disappear if the actual relative humidity at the temperature of reaction is considered. The figures obtained from the isotherms (derived by extrapolation from data at 10 and 37° C) are shown against the individual points in Fig. 3B, and although these values can only be approximate they suffice to show that at high as well as at low temperatures the maximum reaction rate occurs in the region of 65% R.H. and falls away at humidities above and below this value.

#### CHANGES IN COLOUR

The effects of the activity of water at a constant  $p_H$  of 6.3, and of the  $p_H$  of the system at activities of water corresponding to 70% R.H., on the development of a brown discoloration in casein-glucose at 37° C are shown in summarized form in Table II. The development of colour in all cases was preceded by a lag period which became progressively shorter with increasing R.H. or  $p_H$ . This lag period was followed by an initial

TABLE II

THE EFFECT OF ACTIVITY OF WATER AND OF  $p_H$  ON THE DEVELOPMENT OF COLOUR IN CASEIN-GLUCOSE

% R.H. (at $p_H$ 6.3)	Lovibond Y + R units after days at 37° C					
	2	4	8	16	32	64
20	—	—	—	—	—	0.2
40	—	—	—	0.0	0.1	0.6
55	—	—	—	0.1	0.8	1.5
70	—	—	0.0	0.8	1.9	2.7
85	—	0.1	0.8	1.9	3.7	5.0
92½	—	0.1	0.8	1.9	3.3	—
96¼	—	0.1	0.8	2.1	—	—
$p_H$ (at 70% R.H.)						
3.0	—	—	0.1	0.2	0.5	1.1
4.6	—	—	0.2	0.4	1.0	1.9
6.3	—	—	0.1	0.9	1.8	2.6
7.0	—	—	0.3	1.1	2.0	2.9
8.0	—	0.0	0.7	1.6	2.4	3.4
9.0	0.1	0.4	1.2	2.2	3.0	3.8
10.0	0.2	0.8	1.7	2.6	3.4	4.4

rate of development of colour which was more rapid the higher the R.H. or  $p_H$  but which tended to fall away progressively with time, except under very acid conditions ( $p_H$  3 and 4.6) when the rate of increase of colour appeared to be almost linear. The development of colour by protein in the absence of sugar under the conditions of the storage experiments was negligible.

The colour data show no sign of the comparatively sharp maximum in initial reaction velocity observed in the region of 65 or 70% R.H. for the disappearance of free amino-N (Fig. 2). Instead, colour development was approximately twice as rapid at 85, 92½ and 96¼%, the highest humidities at which it could be measured, as at 70% R.H. The measurement of colour became increasingly less satisfactory at humidities higher than 85%, owing to changes in physical structure of the material leading to shrinking and 'stickiness'.

Table II shows that at 37° C and 70% R.H. the rate of discoloration increased continually with increasing  $p_H$  from 3 to 10. At 55% R.H. results were generally similar, the rates at  $p_H$  3, 6.3 and 10 being of the order of 45, 65 and 80% respectively of the corresponding rates at 70% R.H.

The effect of temperature cannot be reported in detail, the different physical states of the samples after the various temperature treatments rendering accurate colour comparison difficult. It was evident, however, that the progressive increase in rate of colour development with increasing water content occurred at all temperatures, the samples containing 13.9% water discolouring more rapidly than those with 10.3 and 6.0% even at 90° C, where the 6% sample showed the most rapid loss of amino-N. At a constant relative humidity of 70% the temperature coefficient was of the same order as that for the disappearance of the amino-N, and the initial lag period persisted at all temperatures. There was some indication, however, that the temperature coefficient was slightly higher than for the amino reaction, and that the lag period was relatively slightly less at the higher temperatures.

#### CHANGES IN SOLUBILITY

In the case of the reaction between reducing sugars and dialysed milk proteins at 37° C<sup>18</sup> it was observed that after a brief initial lag period the protein rapidly became insoluble. The casein-glucose complex on the other hand has a definite affinity for water even when deterioration is marked and considerable colour has developed. Samples with more than half their free amino-N remaining were found to be completely soluble at a concentration of 5%, and even after long storage at 37° C the material could still be reconstituted to a viscous gel. After very long storage at high temperatures however (e.g., 80 minutes at 90° C) water imbibition was slow even in the presence of acid, and for practical purposes the material could be regarded as being insoluble. The relationship of the development of insolubility to the amino-reducing sugar reaction is being further investigated.

#### DISCUSSION

In all of the experiments it has been considered advisable to base conclusions mainly on the earlier stages of the reaction, owing to the probability of partial destruction of the glucose by side or consecutive reactions on continued storage, and to the

possibility of interaction between protein amino groups and sugar degradation products.

The most striking feature of the reaction between casein and glucose appears to be the nature of its dependence on the activity of water in the system, whereby a maximum rate is observed at a moisture content corresponding to an atmospheric humidity in the region of 65 or 70%, while the change is very slow at very low or very high humidities. Since a mild dehydration treatment only could be applied to the casein-glucose mixture it seems quite likely that the small initial reaction observed at 0% R.H. (Fig. 2) was due to water still retained by the protein and that the reaction rate in the absence of moisture would be zero. As the water content increased the rate of the initial reaction and the extent of the change before the reaction slowed down or stopped both increased. After passing the optimum R.H. the initial rate of reaction fell away again quite sharply, but the level of amino-N at which the reaction slowed down or stopped could not be ascertained in this case because of the onset of microbial decomposition.

The relative humidity at which the reaction rate reaches a maximum corresponds with the end of the approximately linear portion of the adsorption isotherm, above which the curve swings steeply upwards (Fig. 1). BULL<sup>15</sup> has suggested, on the basis of the general multilayer adsorption theory of BRUNAUER, EMMETT, AND TELLER<sup>19</sup>, that this point on the isotherm of proteins represents the completion of a double layer of water molecules between the protein planes, and probably also the point at which the exposed polar groups of the protein have become saturated with water. Under these conditions the force of attraction between the protein molecules will be greatly decreased and their capacity for movement and re-arrangement increased\*. As the water vapour pressure is further increased the protein will tend to go into solution. MELLON, KORN, AND HOOVER<sup>18, 20</sup> have recently attempted to carry the matter further by determining the proportion of the bound water which is associated with the free amino groups of isoelectric casein. They conclude that at very low water vapour pressures (0-6% R.H.) one molecule of water is probably held between two amino groups. As the water vapour pressure increases the quantity of water held by the amino groups increases linearly to reach saturation of the hydrogen bonding capacity at about  $2\frac{1}{2}$  molecules, per amino group at 60-70% R.H. Above 70%, and particularly above 80% R.H. there is a rapid increase in the water adsorbed on the amino groups, probably by condensation of water on the water molecules already bound. A fraction varying from one quarter to one third of the total water held by the protein at various humidities was considered to be associated with the amino groups, but this included none of the water responsible for the phenomenon of hysteresis.

On the basis of these data it is not difficult to account for an increasing rate and extent of the reaction between casein amino groups and glucose with increasing R.H. up to 65 or 70%, but less easy to understand the marked falling off in reaction rate as

\* In this respect the work of BARKER<sup>21</sup> on the effect of relative humidity on the temperature of denaturation of egg albumin, and on the effect of denaturation on the water relations of albumin is of interest. BARKER equilibrated his samples of egg albumin to a wide range of relative humidities at room temperature, heated them in sealed tubes at 60 to 160° C for 10 or 60 minutes and deduced a straight line relationship between the temperature required for denaturation and the relative humidity. The samples were therefore heated at constant moisture content and, if allowance be made for the large effect of the high temperatures used on the equilibrium R.H. of the system, it becomes clear that denaturation only occurred above 65-70% R.H. Recalculation of the data in detail has not, however, been attempted, since no account appears to have been taken in the experiments of loss of water into the free space above the samples, which may have influenced the results seriously at the higher temperatures.

the humidity continues to increase beyond this point. It may be that a simple dilution effect is coming into play after sufficient water has been added to bring all the glucose into solution, or that an increasing thickness of aqueous film is tending to keep apart amino and potential aldehyde groups which, while both hydrophylic, have no great affinity for one another as evidenced by their slow and incomplete reaction in aqueous solution. Separate experiments which showed that the rate of reaction at 70% R.H. can be considerably increased by increasing the concentration of glucose above the level employed in these experiments would seem to militate against the simple solubility theory.

The conflicting nature of the conclusions drawn from previous investigations of the effect of  $p_H$  on the reactions between sugars and amino acids or peptides in aqueous solution has already been commented upon (page 314), and the problem is not simplified by the substitution of protein for amino acid and of the "dry" state for aqueous solution. The term  $p_H$  itself is of very doubtful significance in the presence of so little water, and it has been used in the present work only to indicate the reaction of the aqueous dispersion, from which the solid reaction mixture was prepared by freeze drying. Inevitably, this value will tend to fall as the strongly basic amino groups react, and it may be depressed further by the formation of acidic degradation products from the sugar. With protein, however, these changes are comparatively small below  $p_H$  8, and are unlikely to influence the reaction perceptibly in its earlier stages; at  $p_H$  9 and 10 a more marked fall in  $p_H$  commences during drying and continues during storage. The effect of  $p_H$  on the reaction between "dry" casein and glucose differs from that found by FRANKEL AND KATCHALSKY<sup>8</sup> for amino acids or peptides and glucose in aqueous solution in that it proceeds at a very appreciable rate even under quite strongly acid conditions, but is similar in that it increases with increasing  $p_H$  at least as far as  $p_H$  8, and probably up to  $p_H$  10.

The temperature coefficient of 5.4 between 15° and 25° C. for the casein-glucose reaction is somewhat lower than the value of "at least 6" deduced from the earlier experiments on the deterioration of stored milk powder<sup>1</sup>, but this latter figure included a contribution from a change in relative humidity due to the crystallization of lactose, a process which possessed a higher temperature coefficient even than the amino-sugar reaction.

The production of a brown colour from the protein-glucose mixture only when loss of free amino groups occurs, and under conditions where protein and sugar alone are stable, coupled with the existence of a distinct "lag" in the appearance of colour after the fall of amino-N has commenced, support the view that discoloration results from secondary changes in a first formed, colourless protein-glucose complex. The maintenance of a high rate of colour production as the relative humidity is increased above 70%, while the primary reaction between the amino groups and glucose is slowing down, suggests further that increasing concentrations of water considerably beyond 70% R.H. have an accelerating effect on the secondary reactions leading to discoloration. The mechanism of the production of colour will, however, be considered further in work on the preparation and properties of the casein-glucose complex now in progress.

Technical assistance in this work was given by Mr L. J. PARR AND Mr D. N. RHODES. The work was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

## SUMMARY

1. The reaction between casein and glucose (one molecule per free amino group of the casein) in the "dry" state has been studied by determination of free amino-N and measurement of changes in colour.

2. The rate of loss of amino-N has been found to depend on the following factors:

a) It is powerfully influenced by the activity of water in the system, showing a maximum at water contents corresponding approximately to 65 or 70 % relative humidity, and falling away to low values at very high and very low water contents. This relationship has been found to apply at 37, 70 and 90° C.

b) It increases with pH from low values at pH 3 up to pH 8 at least, and probably up to pH 10.

c) It shows a progressive increase with temperature from 0 to 90° C and, when the relative humidity is kept constant at 70 %, conforms to the ARRHENIUS equation with a  $Q_{10}^{15-25^\circ \text{C}}$  of 5.4.

3. The development of colour increases with increase of water content, pH and temperature.

4. Possible mechanisms relating the effects of water to the multilayer adsorption theory of BRUNAUER, EMMETT, AND TELLER are discussed.

## RÉSUMÉ

1. Nous avons examiné la réaction entre la caséine et la glucose (1 molécule par groupe aminé libre de la caséine) en état "sec" par la détermination de l'azote aminé libre et par le mesurage des changements de couleur.

2. Nous avons observé que la vitesse des pertes de l'azote aminé dépend des facteurs suivants:

a) Elle est puissamment influencée par l'activité de l'eau dans le système. Elle est au maximum quand le contenu aqueux correspond à une humidité relative du 65 ou 70 %; elle est petite quand le contenu aqueux est très élevé et quand il est très réduit. Cette relation a été observée à des températures de 37, 70, et 90° C.

b) Elle augmente avec le pH, de petites valeurs à pH 3, jusqu'au moins à pH 8, sinon à pH 10.

c) Elle augmente progressivement avec la température de 0 à 90° C, et, si l'humidité relative reste fixée à 70 %, elle est conforme à l'équation d'ARRHENIUS avec  $Q_{10}^{15-25^\circ \text{C}}$  de 5.4.

3. La coloration augmente avec l'augmentation du contenu aqueux, du pH et de la température.

4. Nous examinons la question des mécanismes possibles qui mettraient les effets de l'eau en relation avec la théorie de l'adsorption multiples couches de BRUNAUER, EMMETT ET TELLER.

## ZUSAMMENFASSUNG

1. Die wechselseitige Reaktion von Kasein und Glukose (1 Molekül per freie Aminogruppe des Kaseins) in "trockenem" Zustand wurde durch Bestimmung des freien Amino-Stickstoffs und Messung des Farbenwechsels geprüft.

2. Es zeigte sich, dass die Verlustgeschwindigkeit von Amino-Stickstoff von den folgenden Faktoren abhängt:

a) Sie wird stark von der Wasseraktivität im System beeinflusst, erweist sich am grössten, wenn der Wassergehalt 65-70 % relativer Feuchtigkeit entspricht, fällt auf kleine Werte bei sehr hohem und sehr niedrigem Wassergehalt. Dieses Verhältnis zeigte sich bei 37, 70 und 90° C.

b) Sie steigt mit dem pH-Wert an, von niedrigen Werten bei pH 3, bis zu mindestens pH 8, wahrscheinlich bis zu pH 10.

c) Sie steigt progressiv an bei Temperaturzunahme von 0-90° C, und entspricht, wenn die relative Feuchtigkeit konstant bei 70 % gehalten wird, der Gleichung von ARRHENIUS mit  $Q_{10}^{15-25^\circ \text{C}}$  von 5.4.

3. Farbenbildung ist gesteigert bei Zunahme von Wassergehalt, pH und Temperatur.

4. Eventuelle Mechanismen zur Beziehung des Wassereffekts auf die vielschichtige Adsorptionstheorie von BRUNAUER, EMMETT UND TELLER werden erörtert.

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THE SULPHUR METABOLISM OF MOULD FUNGI: THE USE OF  
"BIOCHEMICAL MUTANT" STRAINS OF *ASPERGILLUS NIDULANS*  
IN ELUCIDATING THE BIOSYNTHESIS OF CYSTINE

by

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In 1941, STEINBERG<sup>1</sup> showed that many inorganic sulphur sources (sulphate, sulphite, thiosulphate, etc.) were available for the growth of *Aspergillus niger*. He postulated that the mould synthesized organic sulphur compounds *via* "sulphoxylic" acids. Similar results were obtained by HOCKENHULL<sup>2</sup> with *Penicillium notatum*.

In addition, RIPPEL AND BEHR<sup>3</sup> had shown that *Asp. niger*, when supplied with sulphate, liberated considerable quantities of organic sulphur compounds into the medium. Under certain conditions, *P. notatum* could also be induced to convert a large proportion of sulphate in the medium to an unidentified intermediate (HOCKENHULL<sup>2</sup>).

In the present communication, the use of "biochemical mutant" strains of *Asp. nidulans*, in establishing the feasibilities of certain schemes for the biosynthesis of cystine from sulphate, is described. The principle of the method is that many of the steps in such a biosynthesis may be brought about by specific enzymes. The functioning of each of these may, in turn, depend on the intactness of a gene, or set of genes, governing its initial formation. A strain lacking a particular enzyme function for this reason is termed a "biochemical mutant". For example, strains of an organism capable, say, of converting A to intermediate B and intermediate C to final product D, but not of B to C, may be obtained. If D is an essential metabolite, neither A nor B will support growth. However, C (or any substance X easily converted to C) will support growth. By testing possible compounds, the identity of C may be established. This technique has been developed by BR. BEADLES's group at Stanford and is described more fully in a review by BONNER<sup>4</sup>.

EXPERIMENTAL

*The Organisms Used*

The organisms employed were strains of *Asp. nidulans*. The bright green strain, A 69, was kindly given to me by Mr E. YUILL (The American subculture of this is probably N.R.R.L. 195). Y2 was a yellow X-ray mutant of this from Dr G. PONTECORVO. The author's Ba strain was a glaucous mustard-gas mutant of A 69 remarkable for absence of perithecia, dense sporing and rapid growth. "Cystineless" strain,  $\sigma$ , was obtained by Miss J. LASCELLES from strain Y 2.

*References p. 335.*

## MEDIA

1. *C- medium*: glucose 4 %, Na acetate 0.25 %,  $\text{NaNO}_3$  0.5 %,  $(\text{NH}_4)_2\text{SO}_4$  0.5 %, salts PF 1 %, salts HS 1 % in Manchester tapwater,  $\text{pH}$  to 5.8–6.0 with KOH.

2. *C+ medium*: glucose 4 %, Na acetate 0.25 %,  $\text{NH}_4\text{NO}_3$  0.5 %, cystine 0.025 %, salts PF 1 %, salts HC 1 %, in tapwater,  $\text{pH}$  to 5.8–6.0 with KOH.

3. *CD medium*: as C- with the addition of 0.5 % casein hydrolysate powder (vitamin free, ASHE).

4. *Basal medium*: as C+ without cystine.

5. *Malt wort*: Sp.Gr. 1.040.

6. *Salts HS*:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  5 %,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 %,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  0.2 %,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.2 %,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 %,  $\text{Co}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$  0.2 %,  $\text{H}_2\text{SO}_4$  0.2 % v/v, in distilled water.

7. *Salts HC*:  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  5 %,  $\text{FeCl}_3$  0.5 %,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.2 %,  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  0.2 %,  $\text{ZnCl}_2$  0.2 %,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.2 %, HCl conc. 0.2 % v/v, in distilled water.

8. *Salts PF*:  $\text{NaH}_2\text{PO}_4$  10 %, NaF 0.1 % in distilled water.

All solid media contained 2.5 % agar.

Sterilization was carried out at a steam pressure of 20 lb per sq. in for 15 min unless otherwise stated.

## Production and Isolation of "Cystineless" Mutants

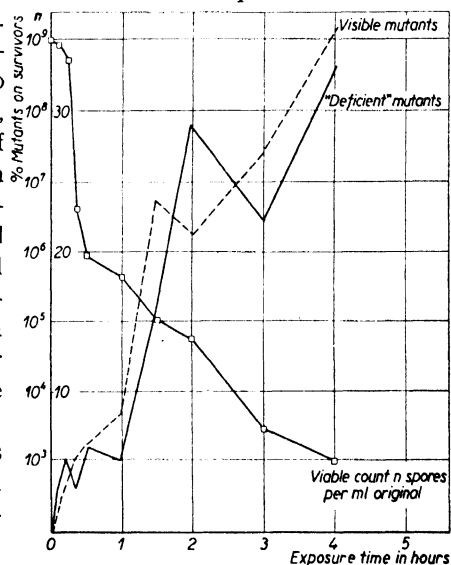
a. *With Mustard Gas in the Vapour Phase*. This technique has already been described (HOCKENHULL<sup>5</sup>).

b. *With Mustard Gas in Aqueous Solution*. Although the vapour exposure method was convenient and clean and gave uniform material which could be stored easily with successful treatments, uniformity between separate experiments was not easily obtained. In addition, the variation introduced by washing off the spores from the discs of felt, the shedding of spores and desiccation during storage and other factors, made difficult the quantitative study of the relation of spore mortality to exposure period. For these reasons, a number of experiments were carried out in which a spore suspension was mixed with mustard gas. This was a modification of the technique of STAHLMANN AND STAUFFER<sup>6</sup> as used with *Neurospora crassa*.

100 ml of a  $10^9$  conidiospore/per ml suspension, in 0.32 M phosphate buffer  $\text{pH}$  6.8–7.0 containing 0.1 % Calsolene oil H.S. (ex I.C.I.Ltd., a wetting-out agent), from a 5–7 day culture of *Asp. nidulans* on malt-wort agar. This suspension was added to 0.25 ml mustard gas ( $\beta\beta'$  dichlorodiethylsulphide) in a dry, sterile, 250 ml conical flask containing about 20 7–9 mm glass balls and incubated at 30° with periodic shaking. At intervals, 2.5 ml of the suspension was withdrawn and added to 100 ml of 0.32 M phosphate buffer  $\text{pH}$  6.8–7.0 containing 0.5 % glycine to inactivate the unused mustard gas.

After standing for about 30 min, 20 ml of this suspension was mixed with 30 ml C- medium in a sterile 1 l conical flask. The whole was incubated for 16 h to initiate germination.

The graph shows the relation between exposure time, survival rate, and the proportion of mutants on the survivors. The morphological mutants were diagnosed by naked-eye examina-



The Effect of Mustard Gas upon an Aqueous Suspension of *Conidiospores* of *Asp. nidulans*. Strain Ba in phosphate buffer  $\text{pH}$  6.9–7.0 at 30° with 0.25 % mustard gas.

tion, the "biochemical" ones by isolation and testing upon C- medium and upon malt-wort agar. For isolation of "cystineless" mutants, survival rates of 50-100 organisms per ml (*ca*  $10^4$  of the original  $10^9$  spores per ml) were desirable. 90-120 min was the usual exposure period.

*c. Isolation of "Cystineless" Mutants.* The filtration technique of FRIES<sup>7</sup> was abandoned in the later experiments on the grounds that, with a 16 h incubation period at 30°, both normal and "cystineless" strains appeared to germinate to the same degree in C- medium. The pregermination was however continued as it appeared to give more uniform results on plating.

The germinated suspension was plated out at the rate of 1 ml per 10 cm petri dish of C+ agar. After incubation at 30° for 2 ½-3 days, separate colonies showing the green of conidiospores in their centres had grown from each viable spore. On this medium, "cystineless" and normal strains could grow well, but strains with other deficiencies could not.

Plates of C- agar (15 ml per 10 cm petri dish) were marked out with 10 mm circles by means of a sterile corkborer. The centre of each of these marked areas was inoculated from a separate colony of treated material. The technique of transfer with a pointed needle (sabre-shaped tip) was critical. Great care was taken to take up very little material and to make sure that spores were not scattered on the marked plate.

Incubation (at 30°) was again carried out for 2 ½-3 days, or until growth in the fastest isolates had reached the edges of the circles. In some circles, it was noted that no growth, or thin spidery mycelium, only had appeared. Such circles were lifted out bodily on a needle and placed on C+ agar. They were inundated with a few drops of a saturated solution of cystine and incubated a further 3 days or more. From those circles upon which growth appeared, inocula were made onto slopes of C+ and C- media. If growth appeared on the former, but failed on the latter, a retest was made to check that the isolate was genuinely "cystineless". The isolated were stored on CD medium with 0.025% added cystine, at room temperature.

13 "cystineless" mutants were obtained from about 9000 isolates with the vapour method and 3 from about 6000 by the liquid-phase method. The apparent difference between the success of the two techniques is largely based upon one extremely successful set of vapour-exposed discs. Therefore, although the author tends to favour the vapour method, no real argument for this choice can be made. All mutant strains were derived from A 69 except  $\xi$  and  $\sigma$  from Y2 and  $\nu$  from Ba.

#### *Substances used in Testing*

Mg ethane sulphinat, from ethyl bromide and SO<sub>2</sub> by the GRIGNARD reaction.

Ba  $\beta$ -sulphopropionate, after KHARASCH AND BROWN<sup>8</sup>.

Ca  $\beta\beta'$ -dicarboxydipropionylsulphide, after CHEYNEY AND PIENING<sup>9</sup>.

Taurine disulphoxide, after CHRISTIANSEN AND DOLLIVER<sup>10</sup>.

$\beta$ -aminoethylmercaptan and bis- $\beta$ -aminoethylsulphidedihydrochloride after MILLS AND BOGERT<sup>11</sup>.

Cysteic acid, after FRIEDMANN<sup>12</sup>.

Cysteine sulphinic acid and cystine disulphoxide, kindly given by Dr MEDES of the Lankenau Institute, Philadelphia. They were made after LAVINE<sup>13</sup>.

Na cysteine S-sulphonate, after CLARKE<sup>14</sup>.

#### *Techniques of Growth-Testing upon various Sulphur Sources*

25 ml melted basal agar were cooled to 60° and poured into a warm, sterile, 10 cm petri dish. 5 ml of a seitz-filtered solution of the sulphur source at p<sub>H</sub> 6-6.5 was added

to bring the final concentration to about 1 mg per ml. The plate was then cooled and cylinders were transferred each to a separate, sterile, plugged 6" × 5/8" tube. They were then individually needle-inoculated with material from the master-cultures of "cystineless" and normal strains, incubated at 30° for 4 days and examined for growth. (Cystine and homocystine were used as steam-sterilized suspensions).

Two completely different tests were carried out with each substance, and at least two replicates per test. Further replication was made if the results appeared uncertain.

In the case of hydrosulphite which was of great theoretical interest, a further series of tests were carried out. Plates of 10 ml of C- medium were poured and sown each with 2 ml of a spore suspension of each organism under test at about 10<sup>7</sup> spores per ml. They were incubated until microscopic examination indicated that germination had taken place. Crystals of hydrosulphite were then added. The growth response was noted after 4 days. The results of these "auxanographic" tests were the same as those by the method described earlier.

The results of the tests are presented in Tables I and II.

TABLE I  
GROWTH OF DEFICIENT STRAINS ON INORGANIC SULPHUR SOURCES

Strain	SO <sub>4</sub> --	SO <sub>3</sub> NH <sub>2</sub> --	S <sub>2</sub> O <sub>6</sub> --	SO <sub>3</sub> --	S <sub>2</sub> O <sub>4</sub> --	S <sub>4</sub> O <sub>6</sub> --	S <sub>2</sub> O <sub>3</sub> --	S <sub>2</sub> --	S--
<i>a</i>	—	—	—	—	—	—	+	—	—
<i>β</i>	—	—	—	—	—	—	+	±	+
<i>γ</i>	—	—	—	++	++	++	++	+	+
<i>δ</i>	—	—	—	++	++	++	++	+	±
<i>ε</i>	—	—	—	—	—	—	+	—	—
<i>ζ</i>	—	—	—	—	—	—	++	—	—
<i>η</i>	—	—	—	++	+	++	++	±	±
<i>θ</i>	—	—	—	—	no test	—	+	+	+
<i>ι</i>	—	—	—	++	++	++	++	+	++
<i>κ</i>	—	—	—	—	—	—	++	±	±
<i>λ</i>	—	—	—	++	+	++	++	—	—
<i>μ</i>	—	—	—	—	—	—	+	—	—
<i>ν</i>	—	—	—	—	—	—	++	—	—
<i>ξ</i>	—	—	—	—	—	—	++	—	—
<i>ο</i>	—	—	—	—	—	—	+	—	—
<i>σ</i>	—	—	—	++	+	++	++	±	±
<i>ν</i>	—	—	—	++	++	++	++	++	+
A 69	++	++	+	++	+	++	++	±	+
Ba	++	++	+	++	++	++	++	++	++

++ vigorous growth

+ moderate growth

± doubtful growth

— no growth

Strains growing on sulphite but not upon sulphate were termed "sulphiteless", while those growing on thiosulphate but not upon sulphite were termed "thiosulphiteless".

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TABLE  
 GROWTH OF DEFICIENT STRAIN

Strain	Na sulphite	L-cystine	Cystine disulphoxide	Cysteine sulphinic acid	Cysteic acid	Ca-bis- $\beta$ -carboxy-ethyl disulphide	Ba- $\beta$ -sulphopropionate	Taurine	$\beta$ -ethylamine sulphinic acid(?)	Bis $\beta$ -ethylamine disulphoxide di-HCl	Bis $\beta$ -ethylamine disulphide di-HCl
$\alpha$	—	++	—	—	—	—	—	—	—	—	—
$\beta$	—	++	—	—	—	—	—	—	—	—	—
$\gamma$	++	++	++	++	++	++	++	++	++	++	++
$\delta$	++	++	++	++	++	++	++	++	++	++	++
$\epsilon$	—	++	±	—	—	—	—	—	—	—	—
$\zeta$	—	++	—	—	—	—	—	—	—	—	—
$\eta$	++	++	++	++	++	++	++	++	++	++	++
$\theta$	—	++	—	—	—	—	—	—	—	—	—
$\iota$	++	++	++	++	++	++	++	++	++	++	++
$\kappa$	—	++	—	—	—	—	—	—	—	—	—
$\lambda$	++	++	++	++	++	++	++	++	++	++	++
$\mu$	—	++	±	+	—	—	—	—	—	—	—
$\nu$	—	++	±	—	—	—	—	—	—	—	—
$\xi$	—	++	—	—	—	—	—	—	—	—	—
$\omicron$	—	++	—	—	—	—	—	—	—	—	—
$\sigma$	++	++	++	++	++	++	++	++	++	++	++
$\upsilon$	++	++	++	++	++	++	++	++	++	++	++
A69	++	++	++	++	++	++	++	++	++	++	++
Ba	++	++	++	++	++	++	++	++	++	++	++

### Genetical Testing of Mutant Strains

Biochemically deficient strains of *Asp. nidulans* have been known for a long time to show the phenomenon of "balanced" heterokaryosis. (Heterokaryosis, the symbiosis of different types of nuclei within the same mould cells, has been described by PONTES CORVO<sup>15</sup> and PONTECORVO AND GEMMELL<sup>16</sup>. It usually occurs after hyphal anastomosis between two different strains). Two strains, each deficient in a different essential metabolite, may by this means live symbiotically upon a doubly deficient medium. The relative numbers of each type of nuclei in the organism will depend upon the rate at which each can cause the formation of the metabolite essential to the other. This ratio is more or less constant for any particular environment, and the heterokaryon will therefore be "balanced" (in contradistinction to the variable heterokaryosis often observed between non-deficient strains).

Suspensions of about  $10^7$  spores per ml were made in C- medium from each strain under test. 2 ml of the suspension from each of the two strains under test for symbiosis were mixed in a 6"  $\times$  1" tube containing a slope of C- medium. After incubation at 30° for 16 h, the suspension was poured away and the residual organisms allowed to

## ORGANIC SULPHUR-SOURCES

ethylamine	Na ethyl sulphate	Na ethane-sulphonate	Mg ethane sulphinate	Diethyl disulphide	"Cyclic" choline sulphate	Acetaldehyde-Na bisulphite	Acetone-Na bisulphite	Formaldehyde-Na-sulphoxylate	Homocystine	Methionine	Cysteine-S-sulphonate	Na sulphite
—	—	—	—	—	—	—	—	—	±	++	+	—
++	—	—	—	—	—	++	++	++	++	++	++	++
—	—	—	—	—	—	—	—	—	—	—	—	—
++	—	—	—	—	—	++	—	—	+	++	++	—
—	—	—	—	—	—	—	—	—	++	++	++	++
++	—	—	—	—	—	+	+	+	++	++	++	++
—	—	—	—	—	—	—	—	—	++	++	++	—
++	—	—	—	—	—	—	++	—	++	++	++	++
—	—	—	—	—	—	—	—	—	±	++	++	—
++	—	—	—	—	—	++	++	+	++	++	+	++
++	++	—	±	±	++	++	++	++	++	++	++	++
++	++	—	—	+	++	++	++	++	++	++	++	++

grow on the slope for another 4 days at 30°. If strong mycelial growth appeared, portions of the growing mycelium were transferred to a second slope of C- medium and again incubated. The presence of continued growth on subculture showed that heterokaryosis had occurred. This finding was checked (to eliminate possibilities of contamination or reversion) by the making of single spore isolations on C+ medium from the non-perithecial portions of the heterokaryons and testing these against C- medium. In all cases of true heterokaryosis, the original strains could be separated out in this way.

The 6 "sulphiteless" strains tested  $\gamma$ ,  $\delta$ ,  $\eta$ ,  $i$ ,  $\lambda$ ,  $v$ ) were found to fit perfectly into three exogamous groups: I,  $\gamma$ ; II,  $\delta$ ,  $i$ ,  $v$  and III,  $\eta$ ,  $\lambda$ .

The 8 "thiosulphateless" strains tested ( $\alpha$ ,  $\beta$ ,  $\vartheta$ ,  $\kappa$ ,  $\mu$ ,  $\nu$ ,  $\xi$ ,  $o$ ) were found to fit into three exogamous groups: I,  $\alpha$ ,  $\beta$ ,  $\nu$ ; II,  $\kappa$ ,  $\mu$ ,  $o$ , and III,  $\vartheta$ ,  $\xi$ , with the following discrepancies:  $\beta \times \kappa$ , ? for -;  $\vartheta \times \nu$ , ? for +;  $\mu \times \nu$  - for +;  $\mu \times \xi$ , ? + (where + = heterokaryosis, - = no heterokaryosis and ? = unreliable results).

7 Combinations between "sulphiteless" and "thiosulphateless" strains were tested and heterokaryosis was obtained in all cases.

The heterokaryon,  $\beta \times \xi$  (green  $\times$  yellow) was maintained for some months on

C- agar. On transferring a small piece of mycelium to C+ agar, it readily segregated into the two parent strains in a typical pattern as described by PONTECORVO AND GEMMELL<sup>17</sup>.

#### DISCUSSION

##### *The State of Reduction at which Inorganic Sulphur is combined with Organic Residues*

Inorganic sulphur-compounds seem to be reduced by the mould at least as far as thiosulphate before being assimilated into organic molecules. The results with sulphide (of which the inconsistency is probably due to the extreme ease with which sulphide is oxidized by atmospheric oxygen) substantially agree with those of STEINBERG<sup>1</sup>, that sulphide is not a precursor in the direct route from sulphate to organic sulphur.

##### *The Reduction of Sulphate*

Sulphate appears to be reduced directly to sulphite. Dithionate is not an intermediate in this process as it behaves wholly as does sulphate. The possibility of a common enzyme for the reduction of both sulphate and dithionate must not be ignored.

##### *The Reduction of Sulphite*

Two paths have been suggested for the reduction of sulphite:

1. The formation of metabisulphite (which, according to ZACHARIASEN<sup>18</sup>, may sometimes exist in the form  $\text{H.SO}_2.\text{SO}_2\text{OH}$ ) with the S-S linkage), its subsequent reduction to hydrosulphite ( $\text{H.SO.SO}_2\text{H}$ ), followed by reduction to thiosulphate ( $\text{HS.SO}_2\text{H}$ ). (c.f., JELLINEK<sup>19</sup> on the electrolytic reduction of sulphite).

2. The direct reduction of sulphite to "sulphoxylate" ( $\text{H}_2\text{SO}_2$ ), with either dimerization of this to form thiosulphate, or, alternatively, enzymic or non-enzymic reaction of this with sulphite to form hydrosulphite and spontaneous reduction of this by "sulphoxylate" to form thiosulphate.

The latter hypothesis is favoured on the following grounds:

1. that, although precise evidence on this point is lacking, the concentration of metabisulphite would be very low indeed under the conditions of the experiment (0.1% aqueous sulphite at a  $p_{\text{H}}$  value about 6).

2. that hydrosulphite behaved exactly as sulphite in the 9 "thiosulphateless" strains tested. This, while not absolutely excluding its intermediacy, does reduce the probability of it very considerably.

##### *The Intermediacy of Thiosulphate*

In the preceding paragraph, it has been assumed that thiosulphate and not sulphoxylate, is the form in which sulphate combines with organic sulphur. Although not completely cogent (for the lack of trials with substances of general formula,  $-\text{S.CH}_2\text{CO.COOH}$ , presents a loophole), the fact that none of the possible intermediates suggested by various possible breakdown routes of cystine and cysteine, could be utilized by any of the thiosulphateless mutants, strongly indicates the irreversibility of such processes. Oxidation of the sulphur of cystine to the sulphinic acid group appears to render it unavailable to "thiosulphateless" strains. Decarboxylation or reductive deamination, with or without oxidation of the sulphur atom, appear also to render the sulphur unavailable to the latter strains.

The observation that the thiosulphate of serine- cysteine S-sulphonate-supports

good growth of the "thiosulphateless" strains, adds a little weight to the above hypothesis. The luxuriant growth of the "thiosulphateless" strains when serine was added together with thiosulphate, is also noteworthy. Chemically, the breakdown of cysteine S-sulphonate in presence of mineral acid to give cysteine and sulphate, or in presence of alkali to give sulphenic acid and sulphite, have been described by CLARKE<sup>14</sup>. In addition, CHALLENGER AND BRISCOE<sup>20</sup> report that, with the mould fungi, *P. brevicaulis* and *Schizophyllum commune*, they have obtained methyl and ethyl mercaptans from the corresponding alkylthiosulphates (Bunte salts). This implies a biologically catalysed fission of the type postulated for cysteine S-sulphonate.

A further point is that no mutants have been isolated which lacked the power to utilize thiosulphate. This makes it extremely unlikely (save if there is more than one route for the synthesis) that more than one enzymic step is involved. It is obvious that the S-sulphonate route entails the least number of possible enzyme-catalysed steps.

Again, the presence of thiosulphate has been noted in many higher organisms, notably by FROMAGEOT AND ROYER<sup>21</sup> in the urine of mammals. The possibility that the non-sulphate fraction in *P. notatum*, noted by HOCKENHULL<sup>2</sup>, may be thiosulphate is now under investigation.

#### *The Utilization of Organic Sulphur Compounds*

The organic compounds tested, with the exception of cysteine S-sulphonate, homocystine and methionine, were not utilized by the "thiosulphateless" strains of the mould.

The ethyl-S compounds (diethyl disulphide, Na ethanesulphonate, etc.) were either not utilized, or only utilized with extreme difficulty, by all strains, including the normal ones. This implies that substitution is necessary before fission of the S-C linkage can occur.

The substituted S-ethyl compounds (with  $\beta$ -carboxy,  $\beta$ -amino or  $\beta$ -carboxy  $\beta$ -amino) were all utilizable by the "sulphiteless" strains while being non-utilizable by the "thiosulphateless" ones. This pointed to a breakdown to yield sulphite.

Recent work by FROMAGEOT, CHATAGNER AND BERGERET<sup>22</sup> indicates that, in mammals, cysteine sulphinic acid is broken down by a sulphinicase to yield alanine and sulphite, is pertinent, especially in view of their claim that the S-C linkage in the compound is very easily broken. It is, however, not easy to see how the action of such an enzyme could explain the breakdown of the *sulphonic* acids tested to yield sulphite. At this stage, therefore, it is unwise to speculate upon possible breakdown mechanisms.

In connexion with this phenomenon, however, it has been noted by HOCKENHULL<sup>2</sup> that *P. notatum* can produce sulphate from cystine. Similar results have been obtained by MÖTHES<sup>23</sup> with *Asp. niger*.

All strains tested grew with homocystine and methionine, especially well with the latter. HOROWITZ<sup>24</sup>, with mutant strains of *Neurospora*, has shown that methionine is synthesized from cysteine *via* L(+)-cystathionine and homocysteine. LAMPEN, ROEPKE, AND JONES<sup>25</sup> found, with *Esch. coli* that, while homocystine or methionine could support growth in all "cystineless" mutants, cystine or cysteine both failed to support growth in some "methionineless" strains. That is to say, in the latter cysteine was primary and that further steps in methionine synthesis were blocked. SIMMONDS<sup>26</sup>, however, has reported a cystineless strain in which none of homocysteine, cystathionine and methionine, supported growth. In this instance the back-reaction to give cysteine from homocysteine appears to have been lost also.



The other miscellaneous compounds tested gave no unexpected results. "Cyclic" choline sulphate behaved exactly as sulphate, the two bisulphite compounds as sulphite, but formosol was not a very good sulphur-source with any of the mutant strains. This last result does not, however, jeopardize the standing of "sulphoxylate" as an intermediate.

### *The Genic Control of Enzymic Reactions*

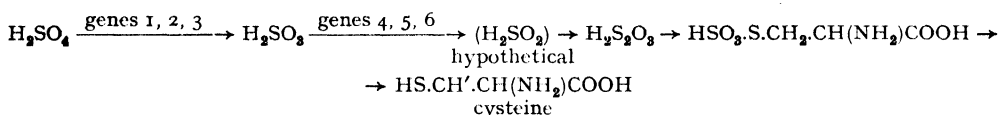
The heterokaryotic phenomena indicate that at least two, and probably three, genes are implicated in each of the changes, sulphate-sulphite and sulphite-thiosulphate.

The author wishes to thank Professor CHALLENGER and Dr BRISCOE of Leeds for helpful discussion and criticism.

### SUMMARY

1. Methods for the isolation of mutant strains with particular biochemical deficiencies are described.

2. "Cystineless" mutants obtained by these techniques have been used in the elucidation of biosynthetic mechanisms. Sulphate appears to be reduced directly to sulphite. It is most probable that sulphite is reduced directly to "sulphoxylate" and does not pass through the metabisulphite-hydrosulphite route. However, it seems that "sulphoxylate" does not combine directly with an organic residue but first dimerized to thiosulphate. The possibility is discussed that cysteine S-sulphonate (serine thiosulphate) may be the next step in the synthesis of cysteine, the most simple hypothesis for cystine synthesis to fit the experimental facts is:



3. None of the possible breakdown routes of cystine tested appear to be reversible. The possible products of such breakdowns by: a) oxidation of the sulphur atom stepwise to the sulphonic acid; b) reductive deamination followed by a); c) decarboxylation followed by a); appear to yield sulphite in presence of the mould.

4. Cystine can be formed from methionine or homocystine without prior breakdown to sulphite.

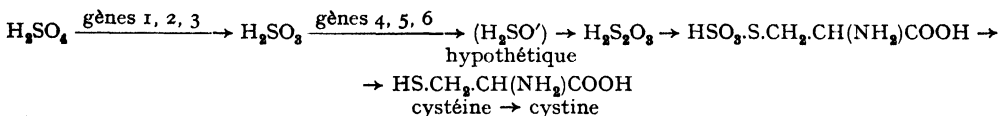
5. The conversion of sulphate to sulphite and that of sulphite to thiosulphate appear in each case to be governed by at least two and probably three genes.

### RÉSUMÉ

1. On décrit deux moyens pour isoler des souches mutantes à déficiences biochimiques spécifiques.

2. On s'est servi de telles souches mutantes "sans-cystine" obtenues par ces moyens-ci pour éclaircir des mécanismes biosynthétiques. Il semble que le sulphate est réduit au sulphite sans intermédiaire. Il est très probable que le sulphite est réduit directement au "sulphoxylate", et ne passe pas par le metabisulphite et l'hydrosulphite au thiosulphate. Néanmoins, il semble que le "sulphoxylate" ne se joint pas à un résidu organique directement, mais, au contraire, il se dimérise pour former le thiosulphate. Il se peut que le cystéine S-sulphonate (thiosulphate de sérine) soit la phase prochaine de la synthèse de la cystine.

L'hypothèse la plus simple qui se rapporte aux faits expérimentaux est:



3. Aucune voie de décomposition de la cystine qui a été étudié ne semble être réversible. Les produits possibles d'une telle décomposition par: a) l'oxydation graduelle de l'atome de soufre jusqu'à l'acide sulphonique; b) la déamination réductive qui est suivie d'(a); c) la décarboxylation qui est suivie d'(a), donnent tous l'acide sulphureux en présence du champignon.

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4. La cystine peut se former à partir de la méthionine ou de l'homocystine sans décomposition préalable au sulphite.

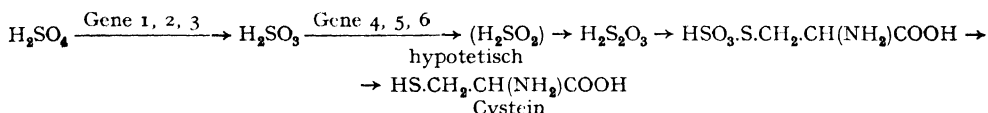
5. Les symbioses hétérokaryotiques indiquent que deux déterminants (gènes) au moins (plus probablement trois) prennent part dans chaque transformation, sulphate-sulphite et sulphite-thiosulphate.

### ZUSAMMENFASSUNG

1. Es werden zwei Methoden beschrieben, um mutierende Stämme mit besonderen biochemischen Mängeln zu trennen.

2. Auf diese Weise isolierte "cystinlose" Stämme wurden zur Aufklärung von biosynthetischen Mechanismen herangezogen. Es scheint, dass Sulfat direkt zu Sulfit reduziert wird. Sulfit wird sehr wahrscheinlich direkt zum "Sulfoxylat" reduziert, und nicht über Metabisulfit und Hydrosulfit zum Thiosulfat. Es scheint aber, dass sich das "Sulfoxylat" nicht direkt mit organischen Resten verbindet, sondern sich zuerst zum Thiosulfat dimerisiert. Möglicherweise ist das Cystein-S-sulfonat (Serinthiosulfat) die nächste Stufe in der Synthese des Cysteins.

Die einfachste Hypothese für die Cystinsynthese die mit den Versuchsergebnissen übereinstimmt, ist die Folgende:



3. Es scheint, dass keiner der möglichen Abbaumechanismen, die der Verfasser untersuchte, reversibel sei. Es zeigte sich, dass ein solcher Abbau a) durch stufenweise Oxydation des Schwefelatoms zur Sulfonsäure b) durch reduzierende Desaminierung gefolgt von a) oder c) durch Decarboxylierung gefolgt von a) in Gegenwart des Schimmelpilzes Sulfit gibt.

4. Cystin kann aus Methionin oder Homocystin ohne vorhergehenden Abbau zu Sulfit entstehen.

5. Die Umwandlungen von Sulfat zu Sulfit und von Sulfit zu Thiosulfat scheinen in jedem Falle von mindestens zwei, wahrscheinlich aber von drei Erbfaktoren bestimmt zu werden.

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## DOSAGE A L'AIDE DE L'ACIDE PERIODIQUE D'UN MÉLANGE DE COLAMINE ET DE SÉRINE

par

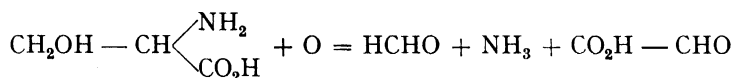
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Les travaux récents ont montré que la constitution des phosphoaminolipides pouvait être plus complexe qu'on ne le pensait généralement.

C'est ainsi que les phospholipides du cerveau fournissent par saponification, à côté des acides glycérophosphoriques, des acides inositolphosphoriques. D'un autre côté, les bases aminées peuvent renfermer de la sérine en plus de la choline et de la colamine. Une détermination de la colamine et de la sérine dans leurs mélanges présente donc un intérêt biologique actuel. Cette détermination a déjà été faite par BURMASTER<sup>1</sup> en oxydant la colamine et la sérine par l'acide periodique, en dosant l'ammoniac libéré et en titrant d'autre part la sérine indépendamment selon VAN SLYKE<sup>2</sup>.

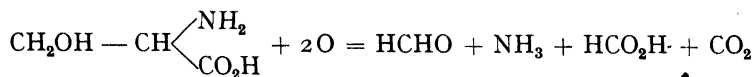
Au cours d'un travail portant sur le comportement de l'acide periodique sur une série de composés hydroxyaminés<sup>3</sup>, travail qui nous a permis de montrer les limites de la réaction, nous avons établi un procédé basé uniquement sur l'emploi de l'acide periodique. Nous avons constaté en effet que l'oxydation de la colamine par l'acide periodique est plus compliquée que la réaction indiquée autrefois par FLEURY ET GUITARD<sup>4</sup>:  $\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\text{OH} + \text{O} = \text{NH}_3 + 2\text{HCHO}$ . En réalité, une molécule de colamine réduit 1.08 molécules d'acide periodique. Ce rapport paraît indépendant des proportions respectives d'amine et d'oxydant. D'autre part, nous avons montré que la sérine est attaquée en deux étapes. La première très rapide fut mise en évidence par NICOLET ET SHINN<sup>5</sup>; une molécule de sérine est oxydée et désaminée en formaldéhyde, ammoniacque et acide glyoxylique.



Différents auteurs ont basé sur cette première étape de la réaction des méthodes de détermination de la sérine en dosant soit le formaldéhyde libéré, soit l'ammoniaque formée. Mais FLEURY ET BON-BERNATETS<sup>6</sup> ont montré antérieurement que l'acide glyoxylique est lui-même oxydé, mais lentement par l'acide periodique, selon la réaction:



Il était donc à prévoir qu'après contact prolongé avec l'acide periodique la sérine serait oxydée selon le schéma:



Nous avons pu en vérifier l'exactitude à  $p_H$  3.0 et  $15^\circ$ . L'étude de l'oxydation en fonction du temps (Fig. 1), indique qu'il se forme rapidement dans un premier temps, deux fonctions aldéhydiques avec réduction d'une molécule d'acide periodique. Par la suite, l'une des molécules d'aldéhyde disparaît, tandis qu'une seconde molécule d'acide periodique est réduite. Ces faits sont conformes à ceux observés au cours de l'oxydation de l'acide glyoxylique. Par ailleurs, au bout de 48 heures, une molécule de sérine oxydée a dégagé 0.95 molécules d'anhydride carbonique (théorie 1.0). Nous avons pensé tout d'abord qu'il serait possible de déterminer la sérine en présence de colamine par dosage de l'anhydride carbonique dégagé. Mais nous avons dû renoncer à ce moyen en constatant que la colamine traitée par l'acide periodique libère toujours du gaz carbonique dont la quantité, bien que faible, n'est pas négligeable.

Nous avons tenté ensuite d'utiliser l'observation suivante relative à l'influence de la réaction du milieu sur la vitesse de l'oxydation par l'acide periodique. Nous avons constaté en effet que la colamine commence à être oxydée d'une façon appréciable à partir de  $p_H$  3.2 tandis que la sérine ne paraît l'être qu'au-dessus de  $p_H$  4.5.

Nous avons alors recherché si, en opérant à  $p_H$  4.0, la colamine serait seule oxydée. Nos essais nous ont montré que pour oxyder totalement la colamine à  $p_H$  4.0, il était nécessaire de prolonger la durée de la réaction d'une façon telle que la sérine subissait un début d'attaque.

Nous avons finalement mis au point une technique basée sur le fait qu'après une oxydation periodique de 48 heures une molécule d'éthanolamine réduit 1.08 molécules d'acide periodique en donnant naissance à une molécule d'ammoniaque.

Dans les mêmes conditions une molécule de sérine donne également naissance à une molécule d'ammoniaque, mais réduit deux molécules d'acide periodique.

Ces deux déterminations expérimentales: acide periodique consommé et ammoniaque formée, permettent de calculer les proportions relatives de colamine et de sérine.

#### MODE OPÉRATOIRE

La prise d'essai doit contenir 25 mg au plus, s'il s'agit de colamine, et 40 mg, s'il s'agit de sérine. Pour les mélanges la teneur maximum sera intermédiaire et variera selon les proportions relatives de ces deux composés.

Deux prises d'essai (5 ml par exemple) sont introduites, l'une dans une fiole jaugée de 100 ml, l'autre dans un erlenmeyer de même contenance — un second erlenmeyer, destiné à contenir l'essai-témoin, reçoit 5 ml d'eau distillée. Dans chacun des trois récipients, nous ajoutons successivement:

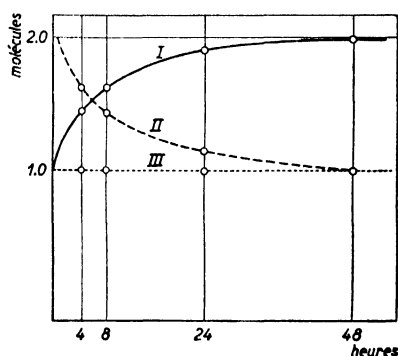


Fig. 1. Evolution de l'oxydation de la sérine par l'acide periodique en fonction du temps

Courbe I: Molécules d'acide periodique réduites par une molécule de sérine

Courbe II: Molécules d'aldéhydes libérés par une molécule de sérine

Courbe III: Molécules d'ammoniac formé par une molécule de sérine

Température:  $15^\circ$

10 ml de solution environ N/10 de periodate de sodium\*,  
5 ml de solution saturée de bicarbonate de potasse,  
20 ml environ d'eau distillée.

Après 48 heures de réaction à la température du laboratoire, nous ajoutons dans les 3 flacons:

15 ml de solution N/10 d'anhydride arsénieux bicarbonaté,  
2 ml de solution à 20% d'iodure de potassium, puis nous laissons en contact 15 minutes.

Nous déterminons d'une part l'acide periodique réduit, de l'autre, l'ammoniac formé.

#### 1. Détermination de l'acide periodique réduit

L'excès d'anhydride arsénieux est titré dans les deux erlenmeyers au moyen d'une solution d'iode 0.1 N sans empois d'amidon.

Soit  $n_1$  ml versés dans l'essai témoin, et  $n_2$  ml versés dans le mélange réactionnel; ces 5 ml du mélange ont donc réduit  $n_1 - n_2$  ml d'acide periodique 0.1 N (0.05 M).

#### 6. Détermination de l'ammoniac libéré

Le volume du liquide, dans la fiole jaugée, est complété à 100 ml. L'ammoniac est titré sur 20 ml du mélange dans l'appareil de PREGL, au moyen d'une solution d'acide sulfurique 0.02 N en présence du mélange rouge de méthyle — bleu de méthylène. Soit  $n_3$  ml utilisés, 100 ml de la dilution, soit 5 ml de la prise d'essai primitive, correspondent à 5  $n_3$  ml d'acide 0.02 N.

#### CALCUL DES RÉSULTATS

Désignons par  $x$  le nombre de ml de colamine 0.1 M contenue dans la prise d'essai, et par  $y$  le nombre de ml de solution de sérine 0.1 M. Nous pouvons écrire:

$$1.08 x + 2 y = \frac{n_1 - n_2}{2}$$

$$x + y = n_3$$

d'où l'on tire:

$$x = \frac{2 n_3 - \frac{(n_1 - n_2)}{2}}{0.92}$$

$$y = n_3 - \left[ \frac{2 n_3 - \frac{(n_1 - n_2)}{2}}{0.92} \right]$$

Or, 1 ml de chacune des solutions 0.1 M contient respectivement:

6.10 mg de colamine

10.51 mg de sérine

\* Solution de periodate 0.1 N:

paraperiodate de sodium $\text{IO}^6\text{Na}^2\text{H}^2$	15 g
solution d'acide sulfurique N	150 ml
eau distillée pour compléter à	1000 ml

La prise d'essai est donc constituée de:

6.1  $x$  mg de colamine

10.51  $y$  mg de sérine.

### RÉSULTATS

L'analyse de divers mélanges de sérine et de colamine a donné les résultats suivants:

TABLEAU I

Le volume des échantillons est de 50 ml

Colamine (mg)		Erreur %	Sérine (mg)		Erreur %
introduite	trouvée		introduite	trouvée	
36.6	37.7	+ 2.5	31.6	30.1	— 4.5
18.3	17.6	— 3.5	31.6	32.2	+ 1.5
0.0	0.0		52.6	53.6	+ 3.7
18.3	19.0	+ 3.7	63.0	62.6	— 0.6
12.1	11.6	— 4.0	31.6	33.2	+ 5.0
24.4	25.3	+ 3.5	63.0	61.6	— 2.0

*Remarques:* 1. Il peut être préférable d'utiliser, au lieu de la quantité d'acide periodique réduit, les quantités d'aldéhydes libérés: les valeurs étant égales, le calcul n'est pas modifié. L'analyse peut alors être effectuée sur des prises d'essai très réduites, la détermination des aldéhydes, comme celle de l'ammoniac, se prêtant à d'excellents micro-dosages bien connus — la spécificité de la méthode serait alors augmentée.

2. Comme il est possible de doser par alcalimétrie seulement la colamine, en présence du mélange rouge de méthyle — bleu de méthylène, dans le mélange sérine-colamine en solutions pures, il suffit de déterminer l'acide periodique réduit global pour obtenir par différence la quantité de sérine contenue dans le mélange. Mais il est évident que cette technique présente moins de spécificité que les deux autres.

3. Il est possible de concevoir une autre variante de la technique, basée sur la détermination de l'acide formique libéré par la sérine après 48 heures d'oxydation periodique, la colamine étant dosée par différence, ou, éventuellement par alcalimétrie.

4. Si le mélange contient des glycols (glycérol, inositol) provenant de l'hydrolyse des phospholipides, il doit être possible, comme des expériences d'orientation semblent le montrer, de doser ces glycols par oxydation periodique, en milieu plus acide que  $p_H$  1.0 (nous avons vu que dans un tel milieu ni la sérine ni la colamine ne sont attaquées). Nous nous sommes bornés simplement à l'étude du mélange des deux amines, qui, d'ailleurs, semblent pouvoir être séparées de l'hydrolysât par des réactifs précipitants sélectifs.

Nous rappelons que la choline, qui peut également accompagner la sérine et la colamine dans les préparations de céphalides, n'est pas oxydable par l'acide periodique<sup>4</sup>.

### RÉSUMÉ

Nous proposons une méthode indirecte du dosage de la colamine et de la sérine dans leur mélange, basée sur la détermination de l'ammoniac dégagé et de l'acide periodique réduit après contact prolongé. Cette méthode permet de doser jusqu'à 25 mg de colamine et 40 mg de sérine. Sa précision est de  $\pm 5\%$ . Elle paraît devoir être applicable aux milieux biologiques.

*Bibliographie p. 340.*

## SUMMARY

An indirect method of estimating colamine and serine in their mixtures is proposed, based on the determination of the ammonia set free and of the periodic acid reduced after prolonged contact. This method allows the estimation of up to 25 mg of colamine and up to 40 mg of serine. This accuracy is  $\pm 5\%$ . It appears to be applicable to biological media.

## ZUSAMMENFASSUNG

Wir beschreiben eine indirekte Bestimmungsmethode von Colamin und Serin in ihren Gemischen; diese gründet sich auf die Bestimmung des abgeschiedenen Ammoniaks und der reduzierten Perjodsäure nach längerer Einwirkung. Es können bis zu 25 mg Colamin und 40 mg Serin bestimmt werden. Die Genauigkeit beträgt  $\pm 5\%$ . Die Methode scheint auch auf biologische Medien anwendbar zu sein.

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## PARTITION CHROMATOGRAPHY OF ENZYMIC DIGESTS OF INSULIN

by

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## INTRODUCTION

Recent work by BUTLER, DODDS, PHILLIPS, AND STEPHEN<sup>1</sup> has shown that crystalline chymotrypsin breaks down crystalline insulin in a reproducible way, about 60% of the total nitrogen becoming soluble in 0.25 N trichloroacetic acid. This 'nonprotein' part was shown by diffusion experiments to have an average molecular weight of about 600, and this value is supported by the amino-nitrogen value of the peptide mixture, so that it consists of some 15 peptides, the average being a tetra or pentapeptide. The VAN SLYKE ninhydrin-CO<sub>2</sub> method<sup>2</sup> showed the presence of one or two free amino-acid residues in the mixture. The number of peptides is therefore not so great as to make their separation seem an impossible task. The methods of paper partition chromatography of CONSDEN, GORDON, AND MARTIN<sup>3,4</sup> have provided for the first time a means of separating minute amounts of amino-acids and small peptides in a precise and complete manner. The separation of di- and tri-peptides has been achieved using the partial acid hydrolysate from Gramicidin S (CONSDEN, GORDON, MARTIN, AND SYNGE<sup>5</sup>).

As far as the author is aware, no work has been published on the examination by this method of the peptides derived from enzymic breakdown of pure proteins.

This paper describes the application of these methods to the separation of the peptides derived from chymotryptic digests of insulin.

## EXPERIMENTAL

The peptide mixtures used were prepared from insulin in the following way. Crystalline insulin (Boots) at a concentration of 5 mg per ml, was digested for 19 to 24 hours at 25° C in pH 8.3 phosphate buffer with  $3.2 \cdot 10^{-4}$  (Trypsin units) of chymotrypsin per ml of digest. The chymotrypsin was prepared by the activation of eight times recrystallized chymotrypsinogen with a very little crystalline trypsin.

The insulin, trypsin and chymotrypsinogen were obtained from beef pancreas. These digestion conditions always produced 50 to 65% of nitrogen soluble in 0.25 N trichloroacetic acid. The whole digest was precipitated by making it up to 0.25 N with trichloroacetic acid by adding one-third of its volume of 1 N trichloroacetic acid, and the precipitate filtered off. The filtrate was then extracted continuously with ether.

Some hydrochloric acid was added towards the end of the extraction to assist the removal of the trichloroacetic acid. The whole extraction occupied about 48 hours and about 95% of the acid was removed, as determined by titration of the extract. The ether



extract, provided entrainment is avoided, contains less than 0.3% of the non-protein nitrogen. The solution of peptides was then neutralized to  $p_H$  7.5 and evaporated down partly below 40° C *in vacuo* and mainly at room temperature *in vacuo* over desiccants, to avoid any undesirable effects of prolonged heating. In all the digests prepared a small precipitate appeared during the ether extractions, being not less than 0.6% of the insulin taken, the upper limit (1–2%) being difficult to assess as the precipitate adhered as a tenacious film to the sides of the extraction apparatus. A similar precipitate was observed when a digest was only 4 times extracted in separating funnels. For the partition chromatography, Whatman No. 1 paper was used throughout. The peptide material was applied in aqueous solution between two pencil lines 0.5 cm apart drawn some 7 cm from one edge of the sheet. About 10 mg peptides was applied along a 50 cm line in this way. The apparatus used was similar to that described by CONSDEN, GORDON, AND MARTIN<sup>3</sup>. Troughs made of polyethylene ('polythene') tubing or from aluminium sheet were found to be suitable and durable. After the run the sheets were dried over an electric hotplate in a good draught. The sheets were then examined in ultraviolet light, (see PHILLIPS<sup>6</sup>) using the light from a "Hanovia" ultraviolet lamp screened by a 1 cm thick Wood's glass filter in a dark room. Thorough drying of the paper beforehand is essential. The fluorescence revealed was generally very faint, and of little use in fixing the position of an amino-acid or peptide spot or band unless the quantity on the paper was large (*e.g.*, 5  $\mu$ g per sq. cm) compared with the amount required to give a ninhydrin colour reaction. However, the method shows up the vagaries of flow of the solvent down a sheet, so that if a one-dimensional run is done with a continuous line or a long row of separate spots of material at the origin, it is possible, developing the edge strips only with ninhydrin, to cut out the bands with some accuracy. The method has also shown that there are highly fluorescent substances in some of the solvents used which travel on two-dimensional chromatograms as spots as small as those obtained with amino-acids. The scope of the paper partition method could thus be extended to further types of substances.

After the ultraviolet examination, the sheet or samples of it are sprayed (avoiding drenching the paper), using an insecticide spray with 0.1% ninhydrin in water-saturated butanol containing 1% of pyridine (see HARDING AND MACLEAN<sup>7</sup>). With the peptide chromatogram considerable heating was required to bring up the colour at all, much of the colour was yellow, and background colour increased rapidly on strongly heating the paper so that this reaction is far less effective here than with amino acids. Using a range of quantities of starting material, the larger amounts, after chromatography and development with ninhydrin not only show increased colour intensities but reveal extra bands as well. One must therefore compromise between taking too much starting material, which will overload the chromatogram, or too little material, which will not be revealed by the rather insensitive peptide ninhydrin-reaction. In some cases the colour development continues or alters in hue long after heating with ninhydrin. Grey colours especially tend to become purple. Such colour changes of peptides with ninhydrin have been noted already by CONSDEN, GORDON, AND MARTIN<sup>4</sup>.

## RESULTS

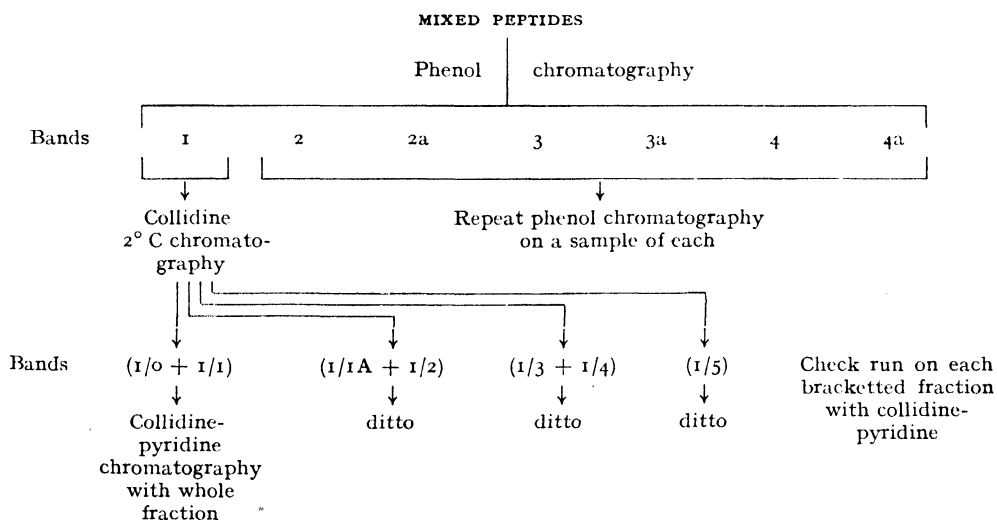
### A. One-dimensional chromatograms of the peptide mixture

Table I gives the scheme of separation applied to the peptide mixture. For the

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first phenol run about 1 g of peptides was chromatographed (about 10 mg per sheet) and edge strips cut for testing. With ninhydrin, four bands appeared: (1)  $R_F$  0.0 to 0.20, (2) 0.25 to 0.50, (3) 0.60 to 0.70, (4) 0.85 to 0.95. The positions and distinctness varied and the gaps were numbered 2a, 3a, 4a. After correlating the sheets the bands were cut out, eluted with water and the eluates evaporated to dryness *in vacuo* below 50° C. Phenol band 1 was then chromatographed with collidine. This was done at 2° C in a refrigerator, to increase the water content and peptide solvent power of the collidine. Ninhydrin treatment of samples of these sheets showed seven bands, (1/0, 1/1, etc.), but some were too close to cut out separately and were eluted in pairs (see Table I). Check runs on samples of these fractions, (1/0 + 1/1), etc., were then made with a collidine-pyridine-water solvent (47:15:38 by volume) which showed that they were complex, some of the components being common to all fractions. As the resolution by collidine-pyridine in the check run seemed to be better than in the bulk collidine run prior to it, the whole of each bracketted fraction (1/0 + 1/1); (1/1A + 1/2); (1/3 + 1/4); (1/5), was run with collidine-pyridine. With ninhydrin they now all gave a similar pattern, differing only in the relative proportions of the bands. The corresponding bands from each were collected together and eluted. Resolution was still incomplete however, as check runs on samples of these eluates showed traces of other bands. Collidine or collidine-pyridine chromatography therefore, can only produce a partial resolution of the mixture.

TABLE I  
SCHEME OF CHROMATOGRAPHY OF THE PEPTIDE MIXTURE FROM INSULIN



When the other phenol bands and gaps, 2, 2a, etc., were checked by running samples of them again with phenol, the same effect was revealed. Band 2 for instance showed the presence of bands 1 and 3 material, and even the 'gap' 2a contained material from bands 1, 2 and 3.

Strip chromatograms were also run using the peptide mixture from several different chymotryptic digests of insulin. These all gave a similar band pattern with phenol, indicating at least a general reproducibility of the enzyme action.

### B. Chromatography with solvents other than phenol

Other solvents were tried after the main work described above. Cyclohexane, benzene, ethyl acetate and *n*-amyl alcohol did not move the material from the application point. Benzyl alcohol and aniline pyridine (1:1) gave only 3–6 cm movement in 18 hours. All solvents were used saturated with water. With *n*-butanol, movement was slow. A 40 hour run with digest C8F produced the following sequence: (1)  $R_F$  0.0 to 0.05, purple and violet ninhydrin colours; (2) 0.09, pale violet; (3) 0.16, grey-purple; (4) 0.22, pale violet; (5) 0.32, pale green; (6) 0.37, pale purple. Band (1) showed three colour changes so that there is evidence of 8 substances here, and the faster materials formed good spots.

*n*-Butanol saturated with an equal volume of five times diluted glacial acetic acid gave the best results obtained so far, and with the same peptide mixture C8F gave the following one-dimensional chromatogram; (1)  $R_F$  value 0.01, pale violet (V) ninhydrin

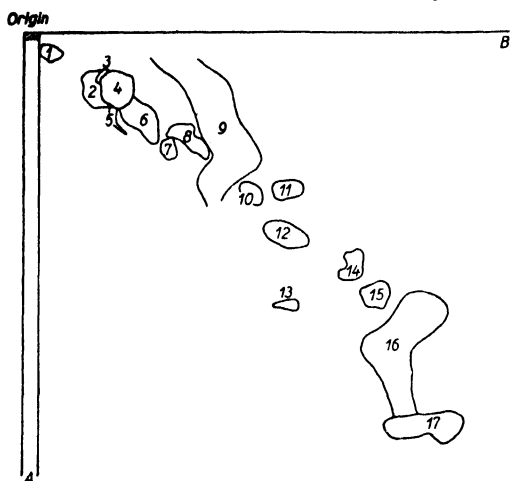


Fig. 1. Ninhydrin colours of the spots as numbered: 1. pale grey; 2. pale blue; 3. blue; 4. blue; 5. violet (V); 6. pale V; 7. very pale V; 8. strong V; 9. V; 10. pale V; 11. pale V; 12. very pale V; 13. pale V; 14. pale grey; 15. pale V; 16. purple; 17. pale purple; 0.5 mg peptide mixture applied at ORIGIN, run in direction A then in direction B with *n*-butanol-acetic acid.

colour; (2) 0.03, pale purple (P); 0.04, yellow; (4) 0.05, blue-grey; (5) 0.07, P; (6) 0.10, pale P; (7) 0.12, pale red; (8) 0.15, pale V; (9) 0.16, pale red; (10) 0.20, pale P. These bands formed a continuous streak but the ninhydrin colour changes were quite distinct. Then followed as separate spots: (11) 0.32, red; (12) 0.52, grey; (13) 0.61, Pale P; (14) 0.73, pale P.

Sometimes the fastest spots gave a green ninhydrin colour. The poor resolution seen in the phenol chromatograms was shown to be largely absent from the butanol-acetic acid chromatograms by running a two-dimensional sheet using butanol-acetic acid as solvent in both directions. Had overlapping occurred in the first run, the subsequent movement in the other direction would have destroyed the spot pattern and revealed streaks of colour with ninhydrin. Fig. 1 shows a copy of the results with digest

C8F, in which 17 spots appeared, with evidence of spread only in spots 9 and 16. The colours on the original faded very rapidly. Two-dimensional chromatograms with phenol or collidine in both directions or with phenol and then collidine were unsuccessful. Ninhydrin revealed only large vague areas of colour.

It is intended to continue the separation with butanol-acetic acid using longer sheets of paper to increase the resolution, and to characterize the individual peptides obtained.

### DISCUSSION

The phenomena described in the experimental part of this paper suggest that with phenol as the chromatographic solvent, several at least of the peptides obtained by the chymotryptic digestion of insulin (averaging tetra-penta-peptides), are spaced out over

a large part of the distance traversed by the solvent. As a result, material eluted from cross-cuts from different parts of a one-dimensional run show identical bands on repeating the chromatography. Collidine and collidine-pyridine as solvents may show similar effects, and it was this difficulty that made the separation procedure both complicated and unsuccessful. No definite cause of the failure of phenol or of the improvement of separation by the subsequent use of *n*-butanol-acetic acid as solvent is apparent though some of the factors involved have been considered.

1. In the first place, the ninhydrin reaction results must be treated with reserve. It would seem that peptides of the size of pentapeptides can form good spots on suitable paper chromatograms and that the formation of broad bands of ninhydrin colour should be taken as evidence of an unsuitable system. The finding that phenylalanine gives a green colour with this reagent when slight heating has been used to dry the chromatograms may have special significance in the case of the peptide mixture used here. The specificity of chymotrypsin according to BERGMANN AND FRUTON<sup>8</sup> requires a phenylalanyl or tyrosyl residue on the carboxyl side of the peptide bond split. The aromatic residue may have its amino group free, but the presence of an  $\alpha$ -carboxyl group in the immediate vicinity inhibits the action of the enzyme. The insulin submolecule of molecular weight 12000 contains 9 tyrosine and 6 phenyl-alanine residues according to CHIBNALL<sup>9</sup>, whilst BRAND<sup>10</sup> finds 8 tyrosine and 6 phenylalanine residues per 12000 weight. Only the positions of two of these are known, the terminal phenylalanyl residues as found qualitatively by JENSEN AND EVANS<sup>11</sup> and confirmed quantitatively by SANGER<sup>12</sup>. BUTLER *et al.*<sup>1</sup> found that for digests of insulin producing 66% non-protein nitrogen, 15-16 peptide bonds per unit of molecular wt 12000 were broken. The accuracy is not high in this measurement, but the agreement between the number of bonds broken and number of aromatic amino acids contained in the submolecule of 12000 may well be significant. This is supported by the finding that the precipitable 'core' of insulin (weight 5000), remaining after digestion, contains 5 aromatic amino-acid residues — 4 tyrosine and 1 phenylalanine, whilst the soluble peptide fraction (total combined weight about 7000) contains 10, 5 tyrosine and 5 phenylalanine (PHILLIPS<sup>13</sup>).

This may mean that after digestion, four aromatic acid residues are at the ends of the four polypeptide chains of the 'core' and that of the 15 or so peptides in the trichloroacetic acid filtrate, 10 have aromatic amino-acids at their carboxyl ends, half of which would then be phenyl-alanyl residues. It is also known that approximately 2 free amino-acid molecules are produced per submolecule of insulin during the chymotryptic digestion. The enzyme specificity (FRUTON AND BERGMANN<sup>14</sup>) makes it likely that the two phenyl-alanine residues with their amino-groups free at the ends of two of the chains of the submolecule, will be split off as free amino acids. The experiment described, using a range of quantities of the peptides with *n*-butanol-acetic acid as chromatographic solvent, yielded several spots with green ninhydrin coloration, which may therefore be indicative of those peptides containing phenylalanine.

2. The conditions under which substances may spread on partition chromatograms have been studied especially by LUGG AND OVERELL<sup>15</sup>. These authors, studying acids such as malic, citric and tartaric, concluded that 'tailing' (the formation of an extended tail behind the main spot) is due to a change in partition coefficient in favour of the aqueous stationary phase as dilution of the material proceeds during the movement, and that in their case this change was due to change in the degree of ionisation. In the

work described here the phenol saturated with water used for the peptide chromatography had a  $p_H$  of 5.4 (glass electrode), which lies within the zwitterionic range of all amino-acids and usual peptides. In n-butanol-acetic acid ( $p_H$  2.8) the  $\alpha$ ,  $\beta$  and  $\gamma$  COOH groups will become far less ionised, favouring non-aqueous phase solubility.

3. The effect of overloading a chromatogram with solutes might have produced the spreading effects seen. With amino acids 'tailing' is rarely seen. The effect of using 80  $\mu g$  of one acid compared with using only 5  $\mu g$  is to increase the dimensions of the spot in a uniform way both in the direction of solvent flow and at right angles to it. This characteristic has recently been made the basis of the quantitative measurement of the amino acid in a spot (FISHER<sup>16</sup>). I have found that phenol-ammonia does occasionally produce a gross elongation of an amino acid spot, especially of aspartic and glutamic acids when the quantity present is high (60–100  $\mu g$ ). The effect is eliminated if the run with *e.g.*, butanol-acetic acid, is done before the phenol run. (It is noteworthy too that glycine and serine are separated better when the solvents are used in this order). With the peptide mixture, using a range of quantities at the starting point varying from 28 to 475  $\mu g/cm$  length of starting line, and phenol as solvent, a similar pattern was produced in all cases, at the highest concentration the bands spreading out and almost merging, while for quantities below 100  $\mu g/cm$ , the faster bands were absent from the developed chromatogram, the ninhydrin reaction apparently being too insensitive. The quantities used in the main chromatography described in this paper were about 200  $\mu g/cm$ . The suitability of the solvent depends partly on the time available to dissolve the solute from the starting-line. This time is increased for instance if the solvent is relatively viscous or the paper inclined so that the solvent speed is reduced or the material placed further down the paper away from the solvent, or by the use of a paper of finer texture. If the critical time is exceeded, the origin becomes equivalent to several separated origins and a streak is bound to result. In practice the time available is very short, for the commonly used solvents are not at all viscous and at the top of the paper at the beginning of a run the solvent moves more rapidly over the paper than at any other time during the development. Thus, for example, on No. 1 Whatman filter paper, phenol and butanol-acetic acid (both solvents saturated with water) took 75 sec and 65 sec respectively to move over a 1 cm wide spot on a level with, and 1 cm from the solvent reservoir, and 3  $\frac{3}{4}$  min and 3 min respectively to move the next 1 cm. Hence if phenol fails as a solvent on this account, n-butanol-acetic acid must have a much more rapid solvent action.

A complication arises in the case described here, since buffers are used in the digestion solution which subsequently go into the trichloroacetic acid filtrate and are chromatographed with the peptides. The proportion of these salts is about 2 mg mainly as phosphate, per 1 mg peptides. One effect of this salt can readily be seen if the solid is shaken up with water-saturated phenol or butanol, when an aqueous salt layer quickly forms. This means that a strong salt solution will collect in the upper part of the paper and cause undersaturation of the solvent passing over it, though the ions of the salts can move down the chromatograms as has been shown by WESTALL<sup>17</sup>.

4. Adsorption does not seem to play a great part in determining the flow of ordinary amino-acids and peptides on paper (CONSDEN *et al.*<sup>3</sup>). It would seem that an adsorption effect can be exhibited with suitable solutes, *e.g.*, N-2:4 dinitrophenyl (DNP) amino-acids. If these acids are run on paper in the usual way but using water or weak aqueous buffers only as solvents, the different DNP-amino acids run as spots at different speeds.

With free amino-acids, or the peptide mixture from insulin under these conditions, all the material runs fast, either at the front or nearly so.

### Acknowledgements

I am grateful to Professor E. C. DODDS AND Dr J. A. V. BUTLER for their help and interest in this work. Thanks are also due to IMPERIAL CHEMICAL INDUSTRIES for financial assistance.

### SUMMARY

The methods of paper partition chromatography have been applied to the separation of the trichloroacetic-acid-soluble peptides from chymotryptic digests of insulin. With phenol and collidine as solvents many of the peptides spread out and could not be separated, but n-butanol-acetic acid as solvent produced several well-defined spots which gave characteristic ninhydrin colours. The significance of these colours with regard to enzyme specificity is discussed and also some of the factors influencing the movement of the peptides on the chromatograms.

### RÉSUMÉ

Les méthodes de la chromatographie de CONSDEN *et al.*<sup>3</sup> sur le papier ont été appliquées à la séparation des peptides de l'insuline (obtenues par l'action du ferment chymotrypsine) qui sont solubles dans l'acide trichloroacétique. Avec le phénol et la collidine comme solvants, plusieurs des peptides se sont répandus sur le papier, et l'on ne peut pas les séparer. Mais le mélange n-butanol-acide acétique comme solvant a produit plusieurs taches bien définies qui ont donné des couleurs caractéristiques avec la ninhydrine. L'importance de ces couleurs à l'égard de la spécificité du ferment et aussi de quelques agents qui influent sur le mouvement des peptides sur le papier est discutée.

### ZUSAMMENFASSUNG

Die Methoden der Chromatographie an Papier von CONSDEN und Mitarbeitern wurden zur Trennung von in Trichloressigsäure löslichen Peptiden aus Insulin, das mit Chymotrypsin verdaut war, angewendet. Mit Phenol und Kollidin als Lösungsmittel, breiten sich viele Peptide weit aus und können nicht getrennt werden; dagegen erhält man mit n-Butanol-Essigsäure mehrere gut definierte Flecke, die mit Ninhydrin charakteristische Färbungen geben. Die Bedeutung dieser Färbungen in Bezug auf die Spezifität des Fermentes, sowie einige, die Verteilung der Peptide auf dem Chromatogramm beeinflussende Faktoren, werden erörtert.

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SUR L'ACTION L-AMINOACIDE OXYDASIQUE DE  
*CL. SPOROGENES* ET DE *CL. SACCHAROBUTYRICUM* EN  
PRÉSENCE D'OXYGÈNE

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AUBEL, ROSENBERG ET DE CHEZELLES<sup>1</sup> ont montré, en étudiant l'influence de l'air sur les anaérobies stricts, la désamination de la L-alanine, à l'air, par *Cl. sporogenes*, et ROSENBERG<sup>2</sup> a constaté la désamination de la L-alanine, à l'air, par *Cl. saccharobutyricum*. Ce même auteur<sup>3</sup> a expliqué cette désamination oxydative par l'intervention de la L-aminoacide-oxydase, avec, pour conséquence, l'influence létale de l'oxygène sous forme d'eau oxygénée. Dans le présent travail nous montrons que les suspensions de *Cl. sporogenes* et de *Cl. saccharobutyricum* lavés sont capables de désaminer un grand nombre d'acides aminés par voie oxydative, en présence d'air, donc dans des conditions non physiologiques pour des anaérobies stricts. Ce fait est d'autant plus remarquable que ces clostridies n'attaquent que très peu d'acides aminés en anaérobiose, la plupart de ceux-ci étant dégradés par la réaction de STICKLAND pour *Cl. sporogenes* et par la transamination via ac. glutamique ou ac. aspartique pour *Cl. saccharobutyricum*<sup>4</sup>. D'une manière générale, il est connu que les clostridies n'attaquent que peu d'aminoacides.

Au cours de ce travail nous avons essayé dix aminoacides avec les suspensions de *Cl. sporogenes* lavé et treize avec les suspensions de *Cl. saccharobutyricum* lavé. Cinq ont été désaminés avec absorption d'oxygène par *Cl. sporogenes* et six par *Cl. saccharobutyricum*. Les bilans d'oxygène absorbé et d'ammoniaque dégagé ont été établis. Les 2-4 dinitrophénylhydrazones de quatre acides cétoniques formés ont été isolées. On a montré la formation de l'eau oxygénée au cours de la désamination oxydative pour *Cl. sporogenes*; pour *Cl. saccharobutyricum*, cette question n'est pas résolue. Nous employons le terme L-aminoacide oxydase pour désigner l'enzyme catalysant la désamination oxydative, avec consommation d'oxygène, des acides aminés naturels par les suspensions des deux clostridies. Nous ferons remarquer que les phénomènes que nous décrivons sont observés avec les bactéries intactes et que l'existence des enzymes invoquées dans le présent travail ne sera complètement justifiée qu'après isolement à l'état pur.

## PARTIE EXPÉRIMENTALE

## Techniques

1. *Préparation des suspensions bactériennes.* Les cultures de *Cl. sporogenes* et de *Cl. saccharobutyricum* sur bouillon de cœur de cheval ou de bœuf non glucosé, âgées de 15–16 heures, sont centrifugées et le culot bactérien lavé une fois avec NaCl à 9<sup>0</sup>/<sub>00</sub>. On recentrifuge et on amène la suspension bactérienne à la dilution désirée avec les solutions de NaCl à 9<sup>0</sup>/<sub>00</sub>.

2. *Acides aminés.* Les acides aminés utilisés sont des produits HOFFMANN-LA ROCHE.

3. *Mesure d'absorption d'oxygène.* Les mesures d'oxygène consommé sont faites par la méthode manométrique de WARBURG. La composition des solutions dans les fioles est indiquée dans les tableaux respectifs. En présence de KCN le logement central contient le mélange de KREBS<sup>5</sup>. La réaction terminée, le contenu de la fiole est déprotéinisée avec un volume égal d'ac. trichloracétique à 8 %, et des prises aliquotes sont prélevées pour le dosage d'ammoniaque, L'ammoniaque est dosé dans l'appareil de PARNAS ET HELLER, par nesslerisation, pour *Cl. sporogenes* et selon la méthode de RAYNAUD ET GROS<sup>6</sup> pour *Cl. saccharobutyricum*.

4. Pour l'isolement et la caractérisation des acides cétoniques, voir le protocole d'expérience.

5. La catalase a été préparée par la méthode de KEILIN ET HARTREE<sup>7</sup>.

## RÉSULTATS

1. *Cl. Sporogenes*

La Fig. 1 montre la consommation d'oxygène en fonction du temps par une quantité croissante de suspension microbienne seule ou en présence d'une quantité constante de substrat (20  $\mu$ M de L-alanine = 448  $\mu$ l). On voit sur les courbes 1, 2 et 3 une diminution rapide de l'absorption de l'oxygène et ceci dès le commencement, ce qui indique une rapide destruction de l'enzyme. En effet, le substrat n'est que faiblement oxydé. La quantité de suspension employée dans les courbes 4 et 5 est suffisante, malgré la destruction rapide, pour que l'acide aminé soit complètement oxydé. C'est la raison pour laquelle nous employons des suspensions denses. Il faut remarquer la quantité importante d'ammoniaque libéré dû à l'autolyse — jusqu' à 29 micromols — avec la suspension la plus dense sans substrat. Celle-ci est probablement responsable de la destruction du système enzymatique en question. On trouvera dans la Fig. 2 les quantités d'oxygène absorbé et d'ammoniaque dégagé en fonction des quantités croissantes de bactéries et en présence de L-alanine. Cette figure montre que même quand le substrat est incomplètement oxydé, le rapport  $O_2/NH_3 = 1$ , comme dans le cas de l'oxydation complète de l'acide-amino.

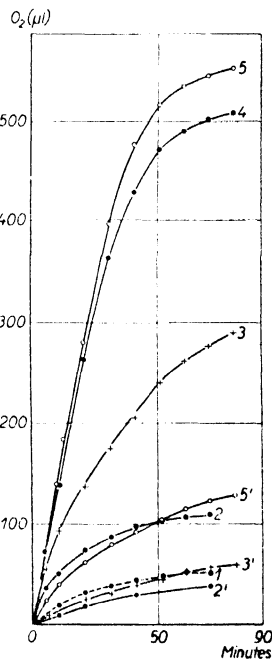


Fig. 1. (*Cl. sporogenes*). Oxydation de la L(+) alanine: 0.2 ml = 448  $\mu$ l en fonction de quantités croissantes de suspension bactérienne. 1) 3 mg (poids sec); 2) 6 mg; 3) 9 mg; 4) 12 mg; 5) 15 mg. Les courbes (2'), (3') et (5') représentent l'absorption d'oxygène sans substrat et correspondent aux courbes 2, 3 et 5, respectivement.



TABLEAU I

(*Cl. sporogenes*)

RAPPORT ENTRE O<sub>2</sub> ABSORBÉ ET NH<sub>3</sub> DÉGAGÉ

Les fioles de Warburg avec logement latéral contiennent 1.0 ml de suspension (15 mg de poids sec); aminoacide, correspondant à 0.2 ml de solution M/10 de l-aminoacide; tampon de phosphate (SØRENSEN), m/15 à pH 7.4. Volume: 3 ml. 0.2 ml KOH à 10 % dans le logement central avec papier filtre. Gaz: air. T = 37°. Durée 100 minutes.

Substrat	O <sub>2</sub> absorbé en micromols	NH <sub>3</sub> dégagé	$\frac{O_2}{NH_3}$
D-alanine . . . .	1.2	1.0	
L-alanine . . . .	18.8	16.7	1.11
DL-alanine . . . .	21.7	20	1.1
L-leucine . . . .	18	22	0.82
DL-isoleucine . .	24	28	0.86
L-valine . . . .	22	26	0.85
DL-méthionine .	6.2	13.3	0.47
L-phénylalanine .	2.7	11.3	
L-tryptophane .	3	4	
L-aspartate . . .	3	4	
L-histidine . . .	0	0	
L-proline . . . .	0	0	

Le Tableau I systématise la relation entre l'oxygène consommé et l'ammoniaque dégagé avec les dix acides aminés essayés.

Les suspensions de *Cl. sporogenes* lavé désaminent oxydativement cinq ac. aminés. Seuls les amino-acides naturels sont métabolisés. Ce sont: l'alanine, la valine, la leucine,

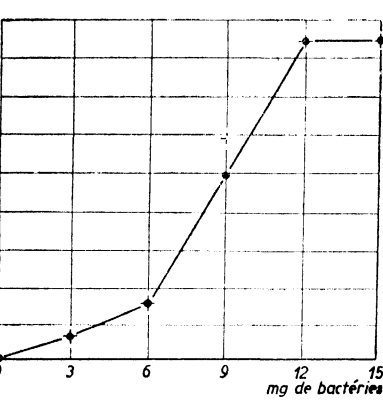


Fig. 2 (*Cl. sporogenes*). Oxygène absorbé (•) et ammoniaque dégagé (+) en millimolécules, en fonction de quantités croissantes de suspension. (O<sub>2</sub> et NH<sub>3</sub> de la suspension sans substrat défalqué)

l'isoleucine et la méthionine. La phénylalanine n'absorbe qu'un faible quantité d'oxygène, mais elle est fortement désaminée. Cette désamination diffère donc de celle des cinq acides aminés ci-dessus. Pour l'alanine, la valine, la leucine et l'isoleucine, le rapport mol oxygène absorbé/mol ammoniaque dégagé = 1. Ces acides aminés absorbent donc 2 atomes d'oxygène par molécule d'ammoniaque. Par contre, la méthionine n'absorbe qu'un atome d'oxygène par molécule d'ammoniaque. Ceci semblerait indiquer qu'il y a différents enzymes responsables pour la désamination des différents acides aminés. Dans le même sens, doit être interprété le fait que le séjour à l'air, entraîne des pertes d'activité notables, mais sélectives. Ainsi, de jeunes suspensions après 6 heures de séjour à la glacière, n'attaquent plus que l'alanine, et non la leucine.

a) *Formation des acides α-cétoniques.* Nous avons isolé les acides α-cétoniques correspondant aux acides aminés suivants: valine, leucine, isoleucine, méthionine.

20 ml d'un mélange contenant 500 micromols d'acide aminé dans du tampon de phosphate M/15 pH = 7.0 et une suspension correspondant à 20 mg d'azote bactérien sont agités à l'air à 37°. Lorsque toute consommation d'O<sub>2</sub> a cessé, on déprotéinise.

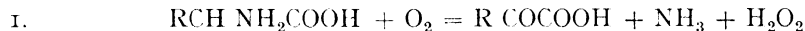
TABLEAU II  
Analyses faites au service de Microchimie de l'Institut Pasteur

2-4 dinitrosophényl hydrazone de	PF	Pf trouvé	N % calc.	N % trouvé	S % calc.	S % trouvé
Ac. $\alpha$ -céto $\beta$ -méthylbutyrique	194°	192°	18.9	18.3		
Ac. $\alpha$ -céto $\beta$ -méthylvalérianique	170°	170°	18.06	17.64		
Ac. $\alpha$ -céto $\gamma$ -méthylvalérianique	155°	155°	18.06	17.85		
Ac. $\alpha$ -céto $\gamma$ -méthiobutyrique	151°	151°	18.07	16.08	9.75	7.18

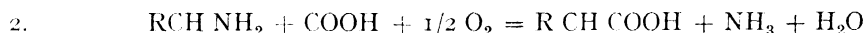
On centrifuge et on filtre; puis on ajoute au filtrat une solution de 2-4 dinitrophénylhydrazine à 0.8 % dans HCl 2N en évitant tout excès par rapport à l'acide cétonique formé. Les hydrazones précipitent presque instantanément. On filtre sur Iéna; le précipité est lavé avec HCl 2N et séché.

En revanche, nous n'avons pas pu isoler l'acide pyruvique provenant de la désamination de l'alanine. La réaction avec la carboxylase ou la précipitation par la 2-4 dinitrophénylhydrazine ont été négatives. Pourtant, nous n'avons pas constaté l'absorption d'oxygène avec l'acide pyruvique + suspension microbienne dans l'appareil de Warburg. Aussi, est-il possible que la disparition de l'acide pyruvique soit due à son oxydation par l'eau oxygénée, produit de la réaction l-amino-acide oxydasique. Et ceci nous amène à considérer la formation de l'eau oxygénée.

b) *Eau oxygénée*. Nous avons vu qu'il y a deux atomes d'oxygène consommé par molécule d'ammoniaque dégagé avec l'alanine, la valine, la leucine et l'isoleucine. L'oxydation des acides aminés se ferait donc d'après la réaction connue:



avec l'amino-acide oxydase du rein ou du foie et en présence de la catalase, la réaction s'écrit:



Il est connu que *Cl. sporogenes* ne contient pas de catalase. Si l'eau oxygénée se forme et ne disparaît pas plus vite par des réactions secondaires qu'elle ne réagit avec la catalase, la consommation d'oxygène doit tomber de moitié, parallèlement avec le quotient respiratoire en présence de la catalase. Aussi avons nous recherché l'influence de la catalase sur la consommation d'oxygène et de dégagement d'ammoniaque avec l'alanine et la leucine. D'après le Tableau III la présence de la catalase n'influence ni l'absorption d'oxygène ni le dégagement d'ammoniaque.

TABLEAU III  
(*Cl. sporogenes*)

INFLUENCE DE LA CATALASE SUR LE RAPPORT OXYGÈNE CONSOMMÉ/AMMONIAQUE DÉGAGÉ

Conditions expérimentales comme dans le Tableau I + 0.1 ml d'une solution concentrée de catalase. Durée: 100 minutes.

Substrat	O <sub>2</sub> absorbé en micromols	NH <sub>3</sub> dégagé	$\frac{\text{O}_2}{\text{NH}_3}$
L-alanine	18	20.5	0.84
L-leucine	16	23	0.7
DL-méthionine	6.4	15.7	0.4

Une légère augmentation d'ammoniaque en présence de catalase s'observe avec la suspension seule comme si la catalase, dans les suspensions sans substrat, favorisait

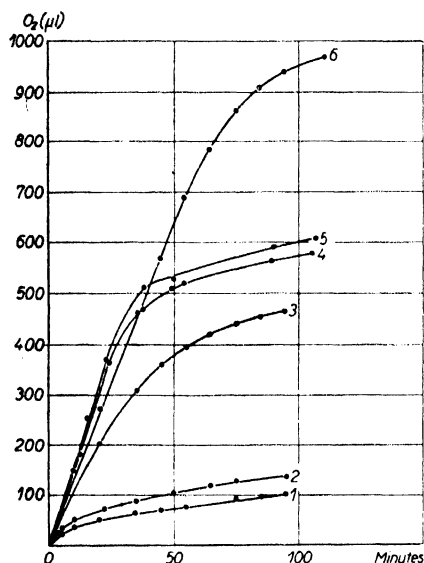


Fig. 3 (*Cl. sporogenes*). Oxydation couplée de l'éthanol par le système L-amino-acide oxydasique des suspensions de *Cl. sporogenes*. Conditions expérimentales comme dans le Tableau IV. Courbe 1) : suspension seule; 2) suspension + 0.2 ml EtOH M/10; 3) comme (2) + 0.1 ml ml de catalase; 4) suspension + 0.2 ml de L (+) alanine M/10; 5) comme (4) + 0.1 ml de catalase; 6) comme (5) + 0.2 ml EtOH M/10

l'autolyse. Le  $QO_2$  avec l'alanine seule est égal à 0.8 et avec l'alanine + catalase à 0.8. Les courbes en présence ou en absence de catalase avec l'alanine, ou avec la leucine ont la même allure. (Courbes 4 et 5 Fig. 3). La catalase n'a donc pas d'action protectrice sur les suspensions de cette clostridie + acide aminé à l'air. De plus, si l'eau oxygénée est formée, la catalase n'agit pas sur elle de façon catalasique.

La détection de très faibles quantités d'eau oxygénée est pourtant possible grâce aux travaux de KEILIN ET HARTREE<sup>8</sup>. L'action peroxydasique de la catalase dans l'oxydation couplée change le rapport  $O_2$  consommé/ $NH_3$  dégagé de 1:1 à 2:1. Ainsi, en ajoutant de l'éthanol au système: alanine + catalase + suspension, nous avons trouvé une augmentation du double de la consommation d' $O_2$ , l'ammoniaque formé restant constant (Tableau IV et Fig. 3).

Il reste à expliquer la consommation importante d'oxygène en présence d'éthanol + catalase + suspension. La suspension seule n'attaque pas l'éthanol, tout en absorbant à elle seule 4 à 5 micromols d'oxygène et en formant, aux dépens de ses produits d'autolyse, l'eau oxygénée, laquelle, en présence de la catalase agit peroxydasiquement sur l'alcool éthylique.

TABLEAU IV

(*Cl. sporogenes*)

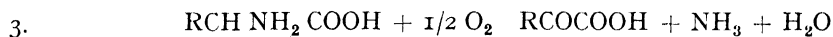
INFLUENCE DE L'ÉTHANOL SUR LA CONSOMMATION D'OXYGÈNE ET SUR LE DÉGAGEMENT D'AMMONIAQUE EN PRÉSENCE DE SUSPENSION BACTÉRIENNE + ACIDE AMINÉ + CATALASE

Flie de Warburg avec deux logements latéraux, contenant 1 ml de suspension 0.2 ml de M/10 acide aminé; 0.1 ml de solution concentrée de catalase, 0.2 ml M/10 EtOH + tampon de phosphate; pH = 7.4; 0.2 ml KOH avec papier filtre. Gaz: air. T = 37°. Durée: 100 minutes.

Substrat	O <sub>2</sub> absorbé en μM		NH <sub>3</sub> dégagé en μM	
	sans EtOH + Cat.	avec EtOH + Cat.	sans EtOH + Cat.	avec EtOH + Cat.
L-alanine	17.5	37.5	20.5	25.3
L-leucine	16	33	20	21

c) *Désamination oxydative de la DL-méthionine*. La méthionine est désaminée oxydativement avec la même vitesse que les quatre acides aminés, mais d'après un schéma différent. En effet, le système méthionine + suspension ne consomme qu'un atome d'oxygène par molécule d'ammoniaque dégagé au lieu de 2 comme avec les autres acides

aminés. La catalase n'a pas d'action, ni sur l'absorption d'O<sub>2</sub>, ni sur le dégagement d'NH<sub>3</sub>. La 2-4 dinitrophénylhydrazone de l'acide cétonique correspondant à la méthionine a été isolée et caractérisée (Tableau II). La méthionine est donc désaminée à l'air par la suspension de *Cl. sporogenes* suivant la réaction 3.



La L-amino-acide oxydase de *Proteus vulgaris* (bactérie aérobie) isolée et étudiée par STUMPF ET GREEN<sup>9</sup> agit également suivant l'équation 3.

d) *Action des inhibiteurs.* Le Tableau V résume l'action de KCN de l'alcool iso-octylique et de NaN<sub>3</sub> sur la désamination oxydative de l'alanine et de la leucine.

TABLEAU V

INFLUENCE DES INHIBITEURS SUR LA DÉSAMINATION OXYDATIVE DE L'ALANINE ET DE LA LEUCINE PAR *Cl. sporogenes* ET PAR *Cl. saccharobutyricum*

	<i>Cl. sporogenes</i>	<i>Cl. saccharobutyricum</i>
	p.c. de l'inhibition	
KCN M/300 . . . . .	100	0
Alc. iso-octylique (Sol. sat.) . . . . .	100	100
NaN <sub>3</sub> M/250 . . . . .	0	0

D'après les travaux de STUMPF ET GREEN, KCN et l'alcool iso-octylique inhibent la L-amino-acide oxydase de *Proteus vulgaris*, mais non celle des tissus animaux. Nous avons obtenu une inhibition de 100% avec les deux inhibiteurs, pourtant, la désamination à l'air de l'alanine et de la leucine par les suspensions de *Cl. sporogenes* se fait avec production d'H<sub>2</sub>O<sub>2</sub>.

## 2. *Cl. saccharobutyricum*

Les suspensions de *Cl. saccharobutyricum* désaminent en présence d'oxygène six acides aminés sur les treize essayés (Tableau VI). Ce sont: la leucine, l'isoleucine la

TABLEAU VI

(*Cl. saccharobutyricum*)

40 micromols d'acide aminé correspondant à la forme L, 1 ml de suspension (15 mg de poids sec). Tampon de phosphate à pH: 7.4. Volume total: 3 ml. Dans le logement central à 2 ml KOH à 10%. Durée d'expérience: 120 minutes.

Substrat	O <sub>2</sub> abs. en micromols	NH <sub>3</sub> dégagé	$\frac{\text{O}_2}{\text{NH}_3}$
L-alanine . . . . .	27	26	1.03
DL-isoleucine . . . . .	18	22.4	0.83
L-leucine . . . . .	27	45	0.60
L-valine . . . . .	9.6	19.2	0.50
DL-méthionine . . . . .	22.7	40	0.51
DL-sérine . . . . .	8.45	11	0.76
L-aspartate . . . . .	11.2	24.2	0.32
L-glutamate . . . . .	5.7	19.4	0.18
L-arginine . . . . .	0	0	
L-histidine . . . . .	0	0	
L-ornithine . . . . .	0	0	
L-proline . . . . .	0	0	
L-glycine . . . . .	0	0	

valine, l'alanine, la méthionine et la sérine. Ici également, il n'y a que la forme naturelle qui soit attaquée.

L'aspartate et le glutamate sont fortement désaminés sans absorption appréciable d'oxygène. Aussi la désamination de ces deux acides aminés se fait-elle par un mécanisme différent de celui des six acides aminés mentionnés plus haut. La même remarque est valable également pour l'histidine.

Le rapport de la consommation d'oxygène sur l'ammoniaque dégagé montre que la valine, la méthionine, la leucine absorbent un atome d'oxygène par molécule d'ammoniaque dégagé, l'isoleucine, l'alanine et la sérine consomment deux atomes d'oxygène par molécule d'ammoniaque dégagé. Nous avons isolé et identifié les mêmes acides cétoniques qu'avec *Cl. sporogenes*. Nous n'avons pas pu isoler l'acide pyruvique provenant de l'alanine ou de la sérine, confirmant ainsi les observations antérieures, — concernant l'alanine — de l'un de nous<sup>2</sup>. Est-il oxydé par l'eau oxygénée problématique, formée au cours de la désamination ou par un autre mécanisme?

a) *Recherche de l'eau oxygénée*. Pour les acides aminés suivants: isoleucine alanine, sérine qui absorbent deux atomes d'oxygène par molécule d'ammoniaque dégagé, il est naturel de supposer la formation de l'eau oxygénée. Nous avons cherché à la mettre en évidence par les mêmes méthodes que pour *Cl. sporogenes*. L'addition de la catalase seule n'a pas donné de résultats, comme pour *Cl. sporogenes* (Tableau VII).

TABLEAU VII  
(*Cl. saccharobutyricum*)

INFLUENCE DE LA CATALASE SUR LE RAPPORT: OXYGÈNE CONSOMMÉ AMMONIAQUE DÉGAGÉ. CONDITIONS EXPÉRIMENTALES COMME POUR LE TABLEAU VI + 0.1 ml D'UNE SOLUTION CONCENTRÉE DE CATALASE

Substrat	O <sub>2</sub> absorbé en micromols	NH <sub>3</sub> dégagé	$\frac{O_2}{NH_3}$
DL-isoleucine . . . . .	23.5	23.2	1.0
DL-méthionine . . . . .	22.4	42	0.53

Le test de KEILIN ET HARTREE, appliqué aux suspensions de *Cl. saccharobutyricum*, ne donne pas un résultat sans équivoque. En effet, l'éthanol seul, ajouté à la suspension, absorbe de l'oxygène et la quantité d'oxygène absorbé avec l'alanine + éthanol + catalase est égal à la somme de l'oxygène absorbé dans les réactions de l'alanine + suspensions et d'éthanol + suspensions respectivement. Cette question n'est donc pas résolue. Il est à remarquer cependant, que la suspension de *Cl. saccharobutyricum* + éthanol consomme la même quantité d'oxygène que la suspension de *Cl. sporogenes* + éthanol + catalase (13 micromols).

b) *Action des inhibiteurs*. Le Tableau V résume l'action des inhibiteurs. Ce qui est à remarquer, c'est que KCN n'inhibe pas l'action amino-oxydasique de *Cl. saccharobutyricum*, tandis qu'il inhibe celle de *Cl. sporogenes*. L'inhibition par l'alcool iso-octylique est de 100%, comme avec l'autre clostridie.

#### DISCUSSION

Nous ne dirons ici que quelques mots sur la formation de l'eau oxygénée. Nous avons montré sa présence dans les suspensions de *Cl. sporogenes* au cours de l'action

L-amino-acide oxydasique par le test de KEILIN ET HARTREE. Nous traiterons ce sujet dans un prochain mémoire avec ses conséquences sur le problème général de l'action léthale de l'oxygène sur les clostridies, comme l'un de nous l'a déjà fait<sup>3</sup>.

En ce qui concerne l'action de la catalase, nous avons montré qu'elle n'exerce pas d'action protectrice sur les suspensions, qu'elle n'agit pas catalasiquement sur l'eau oxygénée présente dans les suspensions. Cette non-protection par la catalase en présence de l'eau oxygénée est à souligner. En effet, l'action protectrice de la catalase contre l'eau oxygénée formée ou ajoutée a été observée par différents auteurs. Ainsi d'après SEVAG<sup>10</sup>, FUJITA ET KODAMA<sup>11</sup>, l'addition de la catalase aux suspensions des pneumocoques à l'air, les protège contre l'effet toxique de l'eau oxygénée. LWOFF ET MOREL<sup>12</sup> avec *Proteus vulgaris*, *B. subtilis*, *B. coli* et BIRKENSHAW ET RAISTRICK<sup>13</sup>, dans le cas de *Staphylococcus aureus*, ont mis en évidence l'action protectrice de la catalase.

Cependant, si la catalase n'agit pas catalasiquement dans les suspensions de *Cl. sporogenes*, elle agit peroxydasiquement en oxydant l'éthanol ajouté par H<sub>2</sub>O<sub>2</sub> formée dans l'oxydation primaire des acides aminés. Ce fait soutient les vues de KEILIN ET HARTREE<sup>8</sup> sur la fonction biologique de la catalase dans les oxydations couplées.

Quant à l'enzyme catalysant la désamination oxydative des acides aminés naturels dans les suspensions de *Cl. sporogenes* et *Cl. saccharobutyricum*, il appartient au groupe des L-amino-acide oxydases. Ce groupe comprend :

1. La L-amino acide oxydase du rein et du foie isolée par la méthode de WURMSER ET FILITTI-WURMSER<sup>14</sup>, par BLANCHARD, GREEN, NOCITO ET RATNER<sup>15</sup> et étudiée par ce même groupe de chercheurs.

2. La L-amino-acide oxydase de *Proteus vulgaris*, isolée et étudiée par STUMPF ET GREEN<sup>9</sup>. Celle des tissus animaux oxyde les substrats en consommant une molécule d'oxygène avec formation d'eau oxygénée, tandis que celle de *Proteus vulgaris* ne consomme qu'un atome d'oxygène sans formation d'eau oxygénée.

La différence entre les deux enzymes se manifeste également envers les inhibiteurs. D'après STUMPF ET GREEN, KCN et l'alcool caprylique sont sans action sur la L-amino-acide oxydase des tissus animaux, tandis qu'ils inhibent la L-amino-acide oxydase de *Proteus vulgaris*. Dans les deux cas, le bleu de méthylène peut remplacer l'oxygène comme accepteur d'hydrogène.

En ce qui concerne l'action L-amino-acide oxydasique des suspensions de *Cl. sporogenes*, si elle ressemble avec l'alanine, la leucine, la valine et l'isoleucine, à celle des tissus, l'inhibition de la désamination par KCN et l'alcool iso-octylique, cependant l'en différence. L'action L-amino-acide oxydasique de *Cl. saccharobutyricum* sur l'isoleucine, sur l'alanine et sur la sérine, ressemble plus à celle des tissus, puisque KCN n'a pas d'action inhibitrice, bien qu'elle s'en différencie par l'inhibition par l'alcool iso-octylique. La désamination oxydative de la méthionine par *Cl. sporogenes* ressemble, et du point de vue de l'absorption d'O<sub>2</sub> et de l'action de KCN et de l'alcool iso-octylique, à celle de la L-amino-acide oxydase de *Proteus vulgaris*. La désamination oxydative de la valine, de la leucine et de la méthionine ressemble à celle de la L-amino-acide oxydase de *Proteus vulgaris* en ce qui concerne l'absorption d'oxygène, mais en diffère par la non-inhibition par le cyanure de potassium.

On note une différence quand on remplace l'oxygène par un colorant comme accepteur d'hydrogène. Le bleu de méthylène ( $E'_0 = 0.011$  V à p<sub>H</sub> 7) peut remplacer l'oxygène aussi bien avec la L-aminoacide oxydase des tissus qu'avec celle de *Proteus vulgaris*. Or, d'après les résultats d'AUBEL, ROSENBERG ET DE CHEZELLES<sup>4</sup>, si le bleu de crésyle

( $E'_0 = 0.032$  V à  $p_H$  7) peut remplacer l'oxygène avec les suspensions de *Cl. sporogenes*, il n'en est pas de même pour *Cl. saccharobutyricum*. Les acides aminés se sont montré de faibles donateurs d'hydrogène avec la suspension de cette clostridie.

En résumé, l'action L-amino-acide oxydasique des suspensions de *Cl. sporogenes* et de *Cl. saccharobutyricum* ne cadre, d'une manière complète, ni avec les propriétés de la L-amino-acide oxydase des tissus, ni avec celle de *Proteus vulgaris*.

De plus, l'action L-amino-acide oxydasique des suspensions de *Cl. sporogenes* diffère de celle de *Cl. saccharobutyricum*: 1) par l'inhibition produite par KCN sur la première; 2) par le comportement différent envers le bleu de crésyle comme accepteur d'hydrogène.

Enfin, ni les suspensions de *Cl. sporogenes*, ni celles de *Cl. saccharobutyricum* n'agissent de la même façon sur les différents acides aminés, bien que l'action des suspensions de *Cl. sporogenes* soit plus uniforme. En effet, elles désaminent oxydativement, en consommant un ou deux atomes d'oxygène. Ces faits sembleraient indiquer: 1) qu'il existe d'autres amino-acides oxydases, différentes de celles des tissus et de celles de *Proteus vulgaris*; 2) qu'il y aurait diverses L-aminoacide oxydases pour les différents groupes d'acides aminés.

## RÉSUMÉ

1. Les suspensions de *Cl. sporogenes* et de *Cl. saccharobutyricum*, à l'air, sont capables de désaminer oxydativement un grand nombre d'acides aminés.
2. Seules les formes naturelles des acides aminés sont attaquées.
3. Avec *Cl. sporogenes* le rapport mol.  $O_2$  absorbé: mol  $NH_3$  dégagé est égal à un pour l'alanine, la valine, la leucine et l'isoleucine. Le même rapport est égal à 0.5 pour la méthionine.
4. Avec *Cl. saccharobutyricum* le rapport mol  $O_2$  absorbé: mol  $NH_3$  dégagé est égal à un pour l'alanine, la leucine, la sérine et à 0.5 pour la valine, la leucine et la méthionine.
5. Les 2-4 dinitrophénylhydrazones, des acides  $\alpha$ -cétoniques correspondant aux acides aminés suivants: valine, leucine, isoleucine, méthionine ont été isolés et identifiés. L'acide pyruvique, provenant de l'alanine ou de la sérine, disparaît dans des réactions secondaires.
6. La formation de l'eau oxygénée a été démontrée avec les suspensions de *Cl. sporogenes* par le test de KEILIN ET HARTREE (oxydation couplée). Pour *Cl. saccharobutyricum*, cette question n'est pas résolue.
7. La catalase ajoutée ne protège pas les suspensions contre l'eau oxygénée; elle n'agit pas catalasiquement, mais d'une manière peroxydasique.
8. Le système enzymatique catalysant l'action L-aminoacide oxydasique est discuté.

## SUMMARY

1. Suspensions of *Cl. sporogenes* and of *Cl. saccharobutyricum* are capable, in air, of deaminating oxydatically a great number of amino-acids.
2. Only the natural forms of amino-acids are attacked.
3. With *Cl. sporogenes* the ratio mols oxygen absorbed: mols ammonia given off is equal to one for alanine, valine, leucine and isoleucine. This ratio is 0.5 for methionine.
4. With *Cl. saccharobutyricum* the same ratio is equal to one for alanine, leucine and serine, and to 0.5 for valine, isoleucine and methionine.
5. The 2-4 dinitrophenylhydrazones of the keto-acids corresponding to the following amino-acids: valine, leucine, isoleucine, methionine, were isolated and identified. The pyruvic acid formed from alanine or serine disappears in secondary reactions.
6. The formation of hydrogen peroxide was demonstrated with suspensions of *Cl. sporogenes* using the KEILIN-HARTREE test (coupled oxidation). This question is not cleared up for *Cl. saccharobutyricum*.
7. The catalase added does not protect the suspensions from hydrogen peroxide; it does not act as a catalase but as a peroxidase.
8. The enzymatic system which catalyses the L-amino-acid oxidase action is discussed.

## ZUSAMMENFASSUNG

1. Suspensionen von *Cl. sporogenes* und *Cl. saccharobutyricum* können an der Luft eine grosse Zahl von Aminosäuren oxydativ desaminieren.
2. Nur die natürlichen Formen der Aminosäuren werden angegriffen.
3. Mit *Cl. sporogenes* ist das Verhältnis des aufgenommenen Sauerstoffes zum abgegebenen Ammoniak in Mol für Alanin, Valin, Leucin, und Isoleucin gleich 1. Für Methionin ist dieses Verhältnis gleich 0.5.
4. Mit *Cl. saccharobutyricum* ist dieses Verhältnis gleich 1 für Alanin Leucin und Serin und gleich 0.5 für Valin, Isoleucin und Methionin.
5. Die 2-4 Dinitrophenylhydrazone der  $\alpha$ -Ketosäuren, welche den folgenden Aminosäuren entsprechen: Valin, Leucin, Isoleucin, Methionin, wurden isoliert und identifiziert. Die Brenzweinsäure, welche aus dem Alanin oder dem Serin stammt, verschwindet in Nebenreaktionen.
6. Die Bildung von Wasserstoffsuperoxyd durch Suspensionen von *Cl. sporogenes* wurde durch den KEILIN-HARTREE'schen Test (gekuppelte Oxydation) nachgewiesen. Für *Cl. saccharobutyricum* ist diese Frage nicht gelöst.
7. Katalase schützt die Suspensionen nicht gegen Wasserstoffsuperoxyd, sie wirkt hier nicht als Katalase sondern als Peroxydase.
8. Das Fermentsystem welches die L-Aminosäureoxydasewirkung katalysiert wird erörtert.

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## THE CHOLINESTERASES OF HUMAN BLOOD

I. THE SPECIFICITY OF THE PLASMA ENZYME AND ITS RELATION  
TO THE ERYTHROCYTE CHOLINESTERASE\*

by

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## INTRODUCTION

It is now well known that the cholinesterase present in human erythrocytes differs in several important respects from that present in human plasma. ALLES AND HAWES<sup>1</sup> were the first to point out that with acetyl choline as substrate, the relation between the initial rate of hydrolysis and the substrate concentration differed for the two enzymes. They also drew attention to certain differences in specificity such as is exhibited, for example, towards acetyl- $\beta$ -methyl choline. Subsequent workers<sup>2, 3</sup> have regarded the differences in specificity as the most striking, and making much of the fact that the plasma enzyme and cholinesterases from certain other sources hydrolyse tributyrin and methyl butyrate, whereas the erythrocyte enzyme, like the enzymes of nervous tissue, electric organs and muscle, does so only slowly<sup>3</sup> or (when freed from accompanying alciesterase) not at all<sup>2</sup>, have referred to the former enzyme as a "non-specific" or "unspecified" esterase<sup>3, 4</sup> or "pseudo" cholinesterase<sup>2</sup>, and to the latter as a "specific" or "true" cholinesterase<sup>2, 3</sup>.

Recent work from this laboratory<sup>5, 6</sup> has shown that the alleged "specific" character of the human erythrocyte cholinesterase is founded on a misconception of the actual specificity range of this enzyme, and that it is, in fact, capable of hydrolysing a wide range of aliphatic substrates, provided the configuration of the ester molecule is not too widely different from that of acetyl choline itself. It is thus apparent that the specificity range does not in itself constitute a valid basis for distinguishing between these enzymes. The fact that, for example, the plasma enzyme hydrolyses tributyrin whereas the erythrocyte enzyme does not, no longer appears to be due to an inherent inability of the erythrocyte enzyme to hydrolyse non-choline esters as such, but appears to be because the butyryl radical is not an optimum acyl radical for this enzyme. It is possible that the two enzymes have essentially similar specificity patterns, but that butyrates bear the same relation to the plasma enzyme which acetates bear to the erythrocyte enzyme.

This hypothesis seems even more probable if we consider the specificity data for horse serum cholinesterase, an enzyme generally believed to be closely similar to human plasma cholinesterase, but whose specificity has been much more fully studied. EASSON AND STEDMAN<sup>7</sup>, using a purified horse serum cholinesterase preparation, showed that

\* With *Addendum* by J. M. NISBET.

propionyl choline is hydrolysed about one and three quarters and butyryl choline over twice as rapidly as acetyl choline. Similar results were obtained by GLICK<sup>8</sup>, who showed that the rate of hydrolysis falls again with valeryl and caproyl choline, butyryl choline being thus the most rapidly split of the homologous series of choline esters.

These considerations suggested that it might be worth-while to examine in greater detail the specificity of human plasma cholinesterase. Little has been previously done on the specificity range of this enzyme beyond establishing that tributyrin, triacetin, and methyl butyrate<sup>9, 10, 11</sup> are also hydrolysed by it. This work has been reviewed in one of our previous papers<sup>11</sup> in which, however, we showed that there is a small quantity of a second enzyme in unpurified plasma, which appears to contribute to a small, but significant extent to the hydrolysis of triacetin and tributyrin. In the present investigation we have used a preparation of human plasma in which this second esterase has been removed and have investigated its specificity towards some 40 esters. It may be stated at once that the results support the hypothesis that the essential difference between the plasma and erythrocyte enzymes is not in their specificity range but in the size of the acyl group which is optimal in each case. With both, the specificity data in general support the idea that as far as the alcohol part of the ester is concerned, the closer the configuration approaches that of choline, the greater the rate of hydrolysis.

#### METHODS

*Source of Enzyme.* Sterile human blood with added anti-coagulant was obtained from the National Blood Transfusion Service. The cells were separated by centrifuging and the plasma freed from a small amount of aliesterase by the method elsewhere described by us<sup>11</sup>, or by the method developed for horse serum by STRELITZ<sup>12</sup>, up to the point where the cholinesterase fraction is precipitated with ammonium sulphate in stage 2. The enzyme was finally taken up in water and dialysed to remove ammonium sulphate.

*Estimation of Enzyme Activity.* The rate of hydrolysis of substrates was estimated manometrically<sup>11, 13</sup>. With non-choline esters, the normal technique was to pipette the requisite amount of pure ester into the main compartment of Warburg vessels, then to add enzyme to the side bulb and enough 0.2 % sodium bicarbonate to the main compartment to give a final total volume of 3 ml after tipping. ADAMS' technique<sup>6</sup> was used with a few esters which tended to give anomalous readings with the normal procedure. With choline esters, the ester was usually added to the side bulb and the enzyme to the main compartment. The "effective" concentration of aliphatic substrates (*i.e.*, the concentration that would have been attained if all the ester had dissolved) was 0.1 M, that of choline esters 0.03 M except where otherwise stated. These concentrations were sufficient to saturate the enzyme and the rates of hydrolysis obtained with them are essentially equal to the limiting rates. The enzyme concentration was adjusted to give between 50–200  $\mu$ l CO<sub>2</sub>/30 min. Non-enzymic controls were always included and all data are corrected for non-enzymic hydrolysis.

#### RESULTS

*Evidence for the homogeneity of the plasma cholinesterase preparations.* We have previously shown the presence in unpurified plasma of a small quantity of DFP-insensitive aliesterase<sup>11</sup>. This was shown to be present by (a) the small degree of positive summation invariably obtained in experiments with choline and non-choline esters, (b) the incomplete inhibition of aliphatic ester hydrolysis by a concentration of DFP sufficient to produce complete inhibition of acetyl choline hydrolysis, (c) a small hydrolysis of triolein which was shown by summation experiments to compete with the DFP insensitive part of the aliesterase hydrolysis. Purification eliminates this aliesterase activity. Our purified preparations had no triolein activity; concentrations of DFP producing complete inhibition of acetyl choline hydrolysis also produced complete

inhibition of the hydrolysis of aliphatic esters, and in summation experiments the rate of hydrolysis of a mixture of representative choline and non-choline esters lay between the rates of hydrolysis of the esters measured separately, indicating the presence of a single enzyme<sup>11</sup>. Some typical results are given in Table I. It will be seen that in all but one case, the rate of hydrolysis of the mixed esters lies somewhat below that of the choline ester alone.

TABLE I  
SUMMATION EXPERIMENTS WITH ALIPHATIC AND CHOLINE ESTERS

Concentrations of substrates: choline esters 0.03 M, aliphatic esters 0.1 M. For meaning of code letters see Table III.

Carbon dioxide evolution ( $\mu\text{l}/30 \text{ min}$ )			
(a) choline ester	(b) aliphatic ester	(c) mixed ester	c-a
ACh + TB			
276	126	276	0
272	120	271	— 1
277	132	275	— 2
BzCh + TA			
317	111	313	— 4
316	105	306	— 10
328	109	320	— 8
BzCh + BuBu			
190	118	182	— 8
192	122	186	— 6
186	115	184	— 2

Purified plasma is found to hydrolyse acetyl- $\beta$ -methyl choline slowly (2% of acetyl choline rate), if the concentration of enzyme is raised sufficiently to make the rate measurable. That this activity is genuinely due to the action of the plasma enzyme and not to a small amount of erythrocyte cholinesterase is shown by the results of Table II. Here, butyl caproate has been used as the aliphatic substrate; again no positive summation has taken place.

TABLE II  
SUMMATION EXPERIMENTS WITH ACETYL- $\beta$ -METHYL CHOLINE AND AN ALIPHATIC ESTER  
Carbon dioxide evolution ( $\mu\text{l}/30 \text{ min}$ )

(a) Acetyl- $\beta$ -methyl choline (0.03 M)	(b) Butyl caproate (0.1 M)	(c) Mixed esters	c-b
19	75	66	— 9
21	75	66	— 9

*The specificity pattern of human plasma cholinesterase.* The relation between an enzyme and its substrate is defined by one or more affinity constants and by the rate constant relating the rate of breakdown of substrate to the concentration of enzyme substrate complex. It is the rate constant which is important in specificity studies for

*References p. 366.*

these may be regarded as attempts to answer the questions, "What compounds are attacked by the enzyme?" and "How rapidly are these compounds attacked?" In general the number of substances capable of combining with the active centres of an enzyme will be larger than those actually breaking down; those combining without breaking down act as inhibitors. While a study of inhibitors may yield valuable information about the configuration of the active centres, we are here concerned with specificity in the narrower sense. Absolute values for the rate constants of substrates cannot be determined without a knowledge of the concentration of the active centres of the enzyme; we have accordingly determined the ratios of the limiting velocities of the various substrates to the limiting velocity of acetyl choline under identical conditions of enzyme concentration,  $p_H$  and temperature. These quantities are equal to the ratio of the rate constant of the substrates to the rate constant of acetyl choline. The values obtained for the limiting velocities expressed as a percentage of that of 0.03 M acetyl choline are given in Table III.

TABLE III

SPECIFICITY DATA FOR PURIFIED HUMAN PLASMA CHOLINESTERASE

Figures are limiting rate of hydrolysis expressed as percentage of that of acetyl choline under identical conditions.

Alcohol	Acetates		Propionates		Butyrates	
	Code	Limiting rate of hydrolysis	Code	Limiting rate of hydrolysis	Code	Limiting rate of hydrolysis
Choline	ACh	100			BuCh	210, 206
$\beta$ -Methyl choline	AcMeCh	1.4			BuMeCh§	12.7, 13.2, 12.6
Triglyceryl	TA	13.5, 14, 14.5			TB	45.5, 44, 47.5
Methyl					MeBu	12, 10.5, 12
Ethyl	EtAc	0.8, 0.9	EtPr	2.6, 2.4	EtBu	6.0, 5.0, 6.0
n-Propyl	PrAc	3.0, 2.7	PrPr*	5.0, 5.0	PrBu	12.0, 11.0, 10.5
n-Butyl	BuAc	11.5, 10.5, 10.0	BuPr*	16.0, 16.5, 15.5, 16.0		
n-Amyl	AmAc	8.5, 7.5, 8.0	AmPr	9.5, 9.0, 9.0	BuBu	22.5, 23, 22
n-Hexyl	HxAc*	2.3, 1.8			AmBu	16, 15.5, 15.5
iso-Amyl	isoAmAc	27, 28, 26	isoAmPr	38, 38.5, 36	HxBu*	3.0, 3.4
1:3-Dimethyl-n-butyl					isoAmBu	40, 45.5, 44, 44
2-Ethylbutyl	1:3-diMeBuAc	2.2, 1.0				
Benzyl	2-EtBuAc	5.7				
3:3-Dimethyl	BzAc	3.3, 4.0				
3:3-Methyl	3:3-diMeBuAc†	35, 35.5	3:3-diMeBuPr†	53		
3:3-Ethyl	3:3-MeEtBuAc†	23, 23	3:3-MeEtBuPr†	24.5, 24.5		
	Valerates		Benzoates		Caproates	
Choline						
isoAmyl	isoAmVa*	5.6, 5.6	BzCh	36.5, 36.5, 35.5	isoAmCa*	9.8, 11.4
n-Butyl	BuVa*	12.6, 11.2			BuCa*	4.9, 4.7
n-Amyl	AmVa*	8, 7.3				

\* Prepared in the laboratory

† Kindly provided by Dr BIRCH

§ Kindly provided by Miss NISBET

It will be seen that like the erythrocyte enzyme, the plasma enzyme hydrolyses a wide variety of aliphatic esters at rates which are, in general, less than those of the corresponding choline esters, but which are nevertheless fairly rapid in the case of the more favourable alcohol configurations. In contrast to the erythrocyte enzyme, however, hydrolysis of propionate esters is in each case more rapid than that of the corresponding acetate, and hydrolysis of the corresponding butyrate is more rapid still; this is true

*References p. 366.*

both for choline and non-choline esters. The effect of increasing chain length in the acyl group for five series of esters is brought out in Fig. 1. It will be seen that in each series an increase in length of the acyl chain leads to a progressive increase in the relative rate of hydrolysis up to butyrate, but that further increase in acyl group size, in those esters which we have had an opportunity of examining, leads to a sharp fall in activity. These results are in accord with STEDMAN's and with GLICK's results<sup>7, 8</sup> for the homologous series of *n*-acyl cholines and horse serum cholinesterase. The reason for the abnormally slow rate of hydrolysis of isoamyl valerate (which one would expect to be hydrolysed faster than the corresponding caproate) is unexplained.

These results are in striking contrast to those obtained with erythrocyte cholinesterase where, it will be recalled, the maximum rate of hydrolysis was obtained, in all the series of esters tested, with acetates, and by which butyrates are hydrolysed only very slowly, if at all. These facts provide an explanation of the well known fact that benzoyl choline is hydrolysed by the plasma enzyme but not by the erythrocyte enzyme. The benzoyl group is even larger than the butyryl group so that we should not expect benzoates to have much chance of being hydrolysed by the erythrocyte enzyme.

**Alkyl Specificity.** Fig. 2 illustrates the effect of increasing the length of the alkyl chain in three homologous series of *n*-alkyl esters. As with the erythrocyte enzyme, increase in alkyl chain length up to 4 carbon atoms (*i.e.*, *n*-butyl esters) results in a progressive increase in the relative rate of hydrolysis; further increases in the length of the carbon chain lead to a falling off in activity.

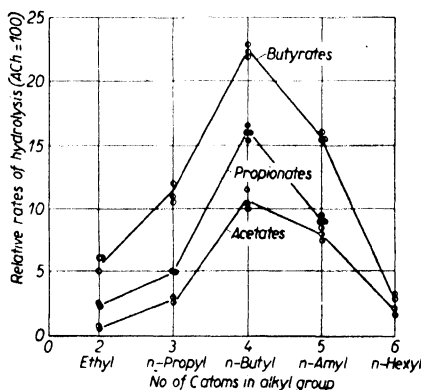


Fig. 2. Effect of chain length of alcohol group on rate of hydrolyses of aliphatic esters by plasma cholinesterase

the choline like configuration is more and more closely approached, the rate of hydrolysis is increased.

References p. 366.

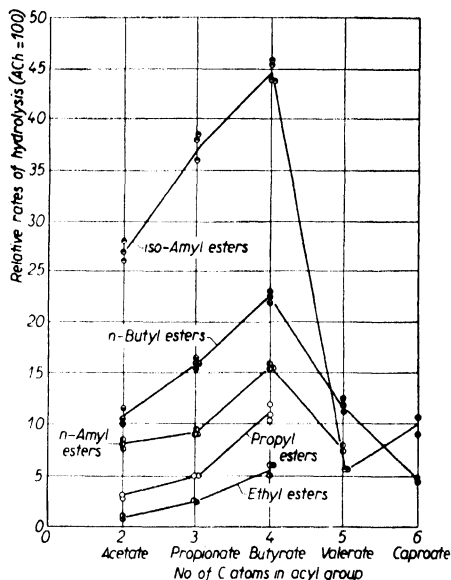


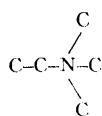
Fig. 1. Effect of acyl group size on rate of hydrolyses of aliphatic esters by plasma cholinesterase

**Effect of Chain branching.** Table IV summarizes the effect of chain branching. Although data for propionates and butyrates are not, in every case, available, the results given leave no doubt that whereas addition of further C atoms to the end of the *n*-butyl chain led (Fig. 2) to a falling off in the rate of hydrolysis, accommodation of these carbon atoms as branches at position 3 (as in isoamyl and 3:3-dimethyl butyl esters) leads to a considerable increase in rate. Once again, as with the erythrocyte enzyme, it is clear that as the

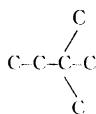
TABLE IV

EFFECT OF CHAIN BRANCHING ON LIMITING RATE OF HYDROLYSIS OF ESTERS BY PLASMA ENZYME

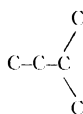
Figures are limiting rates of hydrolysis expressed as percentage of rate of hydrolysis of acetyl choline under identical conditions. (Key to code given in Table III).



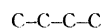
ACh = 100  
BuCh = 206



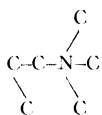
3:3-diMeBuAc = 35  
3:3-diMeBuPr = 53



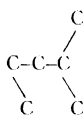
isoAmAc = 27  
isoAmPr = 37  
isoAmBu = 45



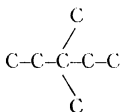
BuAc = 11  
BuPr = 16  
BuBu = 22.5



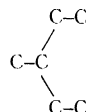
AcMeCh = 1.4  
BuMeCh = 12.8



1:3-diMeBuAc = 2



3:3-MeEtBuAc = 23  
3:3-MeEtBuPr = 24.5



2-EtBuAc = 5.7



BzAc = 3.5

In one respect the "alcohol" specificity of the plasma enzyme differs from that of the erythrocyte enzyme. Chain branching in the carbon atom next to the ester link (C-1 branching) has a far more adverse effect on the rate of hydrolysis by the plasma enzyme than by the erythrocyte enzyme. Table V shows some comparative results for the two enzymes. The effect of chain branching is shown by the ratio of the rate of hydrolysis of the branched chain compound to that of the parent compound. It will be seen that whereas chain branching at C-1 as in acetyl- $\beta$ -methyl choline or 1:3-dimethyl butyl acetate depresses the rate of hydrolysis in the presence of the erythrocyte enzyme by only about one-third, these substrates are hydrolysed by the plasma enzyme at less than 2% and 8% respectively of the rate of their parent compounds. The two enzymes also appear to behave differently with respect to chain branching at the 2 position: in the case of the plasma enzyme substitution of an ethyl group in the 2 position in butyl acetate brings about a considerable reduction in the rate of hydrolysis relative to that of butyl acetate itself, whereas this substitution leads to an enhancement of the rate of hydrolysis in the case of the erythrocyte enzyme. Branching at C-3 leads to an increase in the rate of hydrolysis in both enzymes.

It is thus clear that the behaviour of the "specific" substrate, acetyl- $\beta$ -methyl choline, is not an isolated phenomenon, but is shown by an aliphatic acetate. It was a matter of some interest to see whether the inhibitory effect of C-1 substitution can

TABLE V

THE EFFECT OF CHAIN BRANCHING ILLUSTRATED BY THE RATIO OF THE RATE OF HYDROLYSIS OF VARIOUS BRANCHED CHAIN ESTERS AND THAT OF THE PARENT ESTER

Esters compared	Ratio of rate of hydrolysis		Esters compared	Ratio of rate of hydrolysis (Plasma enzyme)	
	Erythrocyte Enzyme	Plasma Enzyme			
	Branching at C-1				
	$\frac{\text{AcMeCh}}{\text{ACh}}$	0.33 *	$\frac{\text{BuMeCh}}{\text{BuCh}}$	0.062	
	$\frac{1:3\text{-diMeBuAc}}{\text{isoAmAc}}$	0.36			
		0.075			
	Branching at C-2				
	$\frac{2\text{-EtBuAc}}{\text{BuAc}}$	1.3	0.52		
	Branching at C-3				
	$\frac{\text{isoAmAc}}{\text{BuAc}}$	1.5	2.45	$\frac{\text{isoAmBu}}{\text{BuBu}}$	2.0
	$\frac{3:3\text{-diMeBuAc}}{\text{isoAmAc}}$	2.5	1.3		

\* Estimated value for ACh at optimum concentration.

Figures for erythrocyte enzyme from ADAMS<sup>6</sup>.

Figures for plasma enzyme calculated from averaged data given in Table III.

BuMeCh 0.015 M.

For key to code see Table III.

be reduced by replacing the acetate group by a more favourable acyl group, *e.g.*, butyrate. The last column of Table V shows the rates of hydrolysis by the plasma enzyme of butyryl- $\beta$ -methyl choline and isoamyl butyrate relative to their parent compounds. It will be seen that whereas branching in the C-3 position leads to the enhancement of the rate of hydrolysis observed with the acetates and interpreted above as due to a closer approach to the choline configuration, C-1 branching leads to a diminution in the rate of hydrolysis which is again of the same order as that observed with the acetates. Thus, although the  $\beta$ -methyl choline ester/choline ester ratio is 4-5 times greater for the butyrates than for the acetates, we must nevertheless conclude that the effect of C-1 substitution is a general one and largely independent of acyl and alkyl group size.

#### DISCUSSION

Current classifications of the cholinesterases are based on the following assumptions (a) that there are two main types of cholinesterases, those of brain and erythrocytes being prominent examples of the first and those of human and horse serum being prominent examples of the second; (b) that the first type alone have any real claim to be regarded as cholinesterases, the second class hydrolysing choline esters merely as a result of their ability to attack carboxylic esters generally<sup>2, 3, 4</sup>.

The results presented in this paper, taken in conjunction with those already published for the erythrocyte enzyme<sup>5, 6</sup> show that while the number of non-choline esters hydrolysed by the plasma enzyme may well be larger than the number hydrolysed by

the erythrocyte enzyme, the essential difference between them is not primarily one of the specificity range, but lies mainly in the fact that the acyl group which is optimal for the erythrocyte enzyme differs from that which is optimal for the plasma enzyme. Thus, although evidence is forthcoming from other directions against assumption a)<sup>14, 15, 16</sup> our results impugn not it, but assumption b). We agree with ALLES AND HAWES<sup>1</sup> and MENDEL AND RUDNEY<sup>2</sup> that it is unlikely that human plasma cholinesterase contributes significantly to the hydrolysis of acetyl choline in blood and that its physiological rôle is obscure, but the specificity data presented above, by demonstrating that there is a marked preference for those aliphatic substrates which approach most closely the choline configuration, show that the plasma cholinesterase has as much right to be regarded as a cholinesterase as the cholinesterase of erythrocytes, though, to be sure, it is not primarily an "acetyl cholinase". Indeed the results with C-1 and C-2 substitution appear to indicate that the permitted deviation from the choline structure is less in the case of the plasma enzyme. Clearly, much more specificity work needs to be done before a rational and comprehensive scheme of classification of the esterases can be drawn up.

A minor, but interesting point emerging from our specificity work is that the rates of hydrolysis of benzoyl choline by the erythrocyte enzyme and of acetyl- $\beta$ -methyl choline by the plasma enzyme, though very small, are definitely measurable. In each case we have evidence that the hydrolysis is not due to contamination of the enzyme by the cholinesterase of different type. It is unwise, therefore, to attach too much significance to small rates of hydrolysis of these substrates by enzyme preparations or to attempt to use them to measure low concentrations of the enzyme for which they are specific.

The differences between the two cholinesterases of human blood may be summarized as follows:

1. For any given alcoholic group the optimal acyl group for the erythrocyte enzyme is acetate and for the plasma enzyme is butyrate.
2. Both enzymes hydrolyse most rapidly those aliphatic esters which approach most closely the choline configuration, but differ with respect to the effect of chain branching in the carbon atoms of the alcohol adjacent to the ester link. C-1 substitution leads to a fall in the rate of hydrolysis by the erythrocyte enzyme to about one third of the rate of the parent compound in both a choline and an aliphatic ester; in the case of the plasma enzyme the fall is to between 1 and 8% depending on the nature of the parent ester. In the one case of C-2 substitution investigated, an increase in rate was found with the erythrocyte enzyme and a fall with the plasma enzyme.
3. The enzymes show differences in their kinetic behaviour and in the extent to which they are inhibited by certain substances.

We are grateful to the REGIONAL BLOOD TRANSFUSION OFFICER, NATIONAL BLOOD TRANSFUSION SERVICE, for supplies of sterile human blood, and to Dr A. J. BIRCH, Dr H. R. ING and Miss J. M. NISBET of the DYSON PERRINS LABORATORY and the DEPARTMENT OF PHARMACOLOGY, Oxford, for their kindness in synthesizing certain substrates. We are also grateful to Prof. R. A. PETERS for his interest. We are grateful to the MEDICAL RESEARCH COUNCIL (V.P.W.) and to the DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH (D.H.A.) for grants.



## SUMMARY

1. The specificity of human plasma cholinesterase freed from accompanying aliesterase by partial purification has been studied using some 40 choline and non-choline esters.

2. In contrast to the human erythrocyte cholinesterase, the plasma enzyme hydrolyses butyrates most rapidly in any series of esters. The "alcohol specificity" of the enzyme (except in respect of substitution in the carbon atom adjacent to the ester link) is, however, similar to that of the erythrocyte enzyme, aliphatic esters being more rapidly split the more closely they approach the choline configuration.

## RÉSUMÉ

1. La spécificité de la cholinestérase du plasma de l'homme, libérée de l'aliestérase qui l'accompagne par une purification partielle, a été étudiée à l'aide d'environ 40 esters choliniques et non-choliniques.

2. Contrairement à la cholinestérase des érythrocytes de l'homme, l'enzyme du plasma hydrolyse les butyrates avec la plus grande rapidité dans toutes les séries d'esters. La "spécificité vis-à-vis de l'alcool" de l'enzyme (sauf en ce qui concerne la substitution de l'atome de carbone adjacent à la liaison ester) est cependant proche de celle de l'enzyme globulaire, les esters aliphatiques étant d'autant plus rapidement hydrolysés qu'ils se rapprochent plus de la configuration de la choline.

## ZUSAMMENFASSUNG

1. Die Spezifität der menschlichen Plasma-Cholinesterase wurde nach Abtrennung der begleitenden Aliesterase (durch partielle Reinigung) untersucht und 40 Cholin- und Nichtcholinester verwandt.

2. Im Gegensatz zur Cholinesterase der menschlichen Erythrocyten, hydrolysiert das Plasma-enzym Butyrate am schnellsten in allen Serien von Estern. Die Alkoholspezifität des Enzyms (ausgenommen hinsichtlich des Ersatzes des Kohlenstoffatoms nahe der Esterbindung) ist jedoch ähnlich dem Enzym der Erythrocyten. Aliphatische Ester werden um so schneller gespalten, je näher sie der Cholin-konfiguration kommen.

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## ADDENDUM

PREPARATION OF BUTYRYL- $\beta$ -METHYLCHOLINE PERCHLORATE

by

J. M. NISBET

$\beta$ -Methyl choline perchlorate was heated on a water-bath under reflux for three hours with excess redistilled butyryl chloride. The reaction mixture solidified on cooling, and was extracted with dry ether several times and then recrystallized twice from ethanol. Colourless crystals, m.p. 101° C. (Found: C, 41.95; H, 7.70  $C_{10}H_{22}O_6NCl$  requires C, 41.74; H, 7.65 %).

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## THE TERMINAL CARBOXYL GROUPS OF INSULIN

by

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According to SANGER<sup>1</sup> and TISELIUS AND SANGER<sup>2</sup> the insulin sub-molecule of molecular weight 12000 consists of four open peptide chains, connected by the dithio bridges of cystine. Two of these chains end in glycyl residues, the two other having phenylalanine as terminal groups.

As yet there are no indications which amino acid residues are present at the opposite end of these four chains, *i.e.*, those with a free carboxyl group or with a carboxy-amide group. The partial proteolysis under the influence of carboxypeptidase appeared to offer a useful method to tackle this problem. Its use for this purpose has frequently been discussed in literature. In the case of glutathione, the terminal group has actually been determined with the aid of this enzyme<sup>3</sup>.

The difficulties in the evaluation of the results obtained with carboxypeptidase arise from the fact that a sufficient number of examples of its action on simple polypeptides is still lacking. Therefore the general conclusion that carboxypeptidase liberates only amino acid residues with a free carboxyl and a bound  $\alpha$ -amino-group is not fully convincing, and some exceptions to this rule are already present amongst the few peptides investigated. BERGMANN AND HOFMANN<sup>4</sup> observed that carbobenzoxyglycyl-glutamic acid was only slowly attacked, whereas  $\epsilon$ -hippuryllysine is split quite readily, although it has a free  $\alpha$ -amino group.

Furthermore it is not at all a well established fact that carboxypeptidase is able to hydrolyse the terminal groups of intact proteins. Its action on casein, digested with trypsin, is well known and sometimes used for assay purposes, but so far no experiments with an intact native protein have been recorded.

We have now demonstrated that carboxypeptidase attacks insulin, splitting off one or more terminal groups. These have been identified, offering some further insight into the fine structure of this protein.

## EXPERIMENTAL

The insulin used was crystalline material of our own manufacture, which in the air dry state showed an activity of 25 international units per mg. The moisture content was 9%.

The carboxypeptidase was prepared from cattle pancreas according to ANSON<sup>5</sup>. It was recrystallized three times and its activity was checked with chloroacetyl tyrosine. It was stored in the ice box as an aqueous suspension. The course of the hydrolysis was followed by the determination of the free amino groups with the manometric VAN SLYKE apparatus.

The determination of the amino groups liberated offers certain difficulties, because the value for the free amino groups in the intact insulin molecule is dependent on the

experimental conditions. The reaction time is very important as SANGER<sup>1</sup> rightly pointed out, but the temperature at which the reaction takes place is of no less influence. Our zero value, representing the free amino groups of the intact molecule, varied from 6.0 to 8.0 aequivalent  $\text{NH}_2$  per mol insulin of weight 12000. The highest value was obtained at 30°, the lowest at 16° C, both with a reaction time of 5 minutes. As the temperature of the VAN SLYKE reaction chamber cannot easily be controlled, it was decided to take the increase of the amino groups rather than the absolute value as a criterion. As during the course of one experiment the room temperature, which is also the temperature of the reaction chamber, did not vary greatly, amino acid determinations of one run were comparable.

For an experiment 500 mg of the insulin were dissolved in 10 ml of water, by adjusting the  $\text{pH}$  to 7.7 with the aid of 0.1 N NaOH. No buffers were added in order to

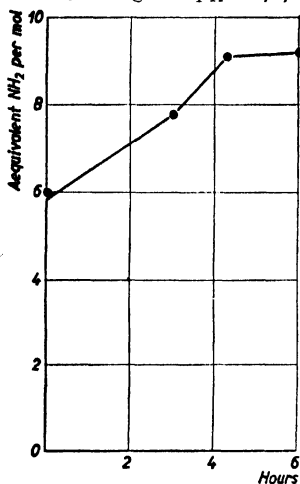


Fig. 1. 500 mg insulin digested with 4 mg carboxypeptidase. Volume 10.2 ml  $\text{pH}$  7.7. Temperature 37°. Abscissae: time in hours. Ordinate: number of  $\text{NH}_2$  groups per submol of insulin (M.W. 12000).

avoid interference in the partition chromatography of the digestion mixture. To this solution 0.2 ml of a suspension containing 4 mg of the carboxypeptidase crystals were added. During the reaction a temperature of 37° was maintained. As represented in Fig. 1, the VAN SLYKE amino nitrogen value of the solution increased steadily during the first 4 hours of the experiment and then slowed down considerably, 3.1 amino groups having been set free. In one experiment the reaction was stopped after 2.6 amino groups had been liberated, by the adjustment of the  $\text{pH}$  to 5. The solution was filtered from the precipitate formed, and subsequently ultrafiltered through a cellophane membrane pretreated with a 100W/V % solution of zinc chloride to increase the porosity.

In the ultra filtrate the total (Kjeldahl) nitrogen amounted to 3.80 mol per mol of insulin employed. 1.24 mol was due to ammonia, and the remaining 2.56 mol agreed well with the direct amino nitrogen determination in the ultra filtrate of 2.6 mol. Hence within the experimental error all amino groups liberated during the proteolysis are accounted for in the ultra filtrate, which, besides some ammonia, does not contain any other form of nitrogen. There-

fore the enzyme actually liberated amino acids from the end of the peptide chain, and no peptides have been formed. The ammonia liberated is not dependent on the enzyme action.

For the analysis of the amino acids present in the ultra filtrate use has been made of partition chromatography on paper. Phenol and collidine have been employed as moving solvents. As suggested by WINSTEN<sup>6</sup>, phenol shaken with saturated NaCl solution has been used in the later experiments, thus avoiding water logging of the paper.

The paper selected after many failures was type V 262 of G. SCHUT, Heelsum, Holland; it gives excellent straight sharp liquid fronts and almost no background colour after spraying with ninhydrin solution.

Only one amino acid, *alanine*, has been detected in the ultra filtrate of this insulin digest. Another digestion was continued for 6 hours, to the point where 3.16 amino groups were liberated according to the VAN SLYKE determination. Now, apart from

alanine, some weak spots appeared. To determine these the solution was concentrated *in vacuo*. As the increased electrolyte content caused some water logging of the paper and an obvious shift of the now very strong alanine spot, the solution was desalted in the apparatus of CONSDEN, GORDON, AND MARTIN<sup>7</sup>, and subsequently analysed with good results. Glycine, valine, the leucine group, and traces of tyrosine and of the mono amino dicarboxylic acid group were detected.

As a further breakdown of the molecule could not reveal anything of interest, the digestion was not continued any further.

A sample of the insulin has been investigated after 1.80 mol of alanine had been liberated. The solution was adjusted to  $p_H$  3 to stop the enzyme action and biologically assayed in rabbits. 15% of the original activity was still left. At  $p_H$  5 no crystal formation was observed, only a heavy amorphous precipitate appeared. A solution of  $p_H$  2.8 in 0.01 N sodium sulphate showed only a slight precipitate after 20 minutes in a boiling water bath. The original insulin under these conditions shows a bulky precipitate after a few minutes' heating.

#### DISCUSSION

From these investigations it appears certain that of the amino acid residues with a free carboxyl group, present in an insulin submolecule, one consists of alanine. One or two of the others remaining might also be alanyl groups, but these experiments cannot bring a clear-cut decision in this respect. An alternative is a di-alanyl-alanine group at the end of one chain and groups which are much more slowly attacked by carboxypeptidase on the other ends. An intermediate configuration, with one alanyl-alanine group and one alanine group on two of the chains, is also possible.

When the alanine has been split off a considerable number of amino acids appear simultaneously in the solution at a greatly reduced rate. The technique did not permit to determine these quantitatively because of the very minute amounts present and it is questionable if it would be useful to explore this phase in more detail, further exact location of the involved amino acids in the chain remaining an impossible task.

The fact that the insulin did not show any tendency to crystallize after a short incubation is not very surprising: the precipitate is certainly not homogeneous, un-attacked insulin and molecules with from one to three alanine groups missing being present simultaneously.

The lack of the property to form a heat precipitate after only a few amino acids have been removed from the chain will have to be taken in account in the explanation of the heat-precipitation of the intact molecule. However, as ANSON<sup>8</sup> rightly pointed out, it is still too early to draw a picture of the denaturation mechanism in detail, and the fact mentioned is difficult to reconcile with any of the existing theories on the subject.

In principle the application of this method to other proteins, in order to elucidate the nature of the terminal group, will be possible. However it should be borne in mind that with insulin circumstances were particularly favourable. In the first place the insulin sub-unit is rather small. Nevertheless it was necessary to detect one amino acid, which, assuming an average molecular weight of 100, could be expected to be present in a quantity of 0.8% of the weight of the protein employed. Secondly it was found that the first three amino acids liberated were identical, making the amount of the amino acid to be analysed at once three times as large as originally anticipated. Thirdly the check

on the course of the enzyme reaction by analysis of total N and amino-N in the filtrate would have been irrelevant had the liberated amino acid contained other than amino nitrogen.

All three factors considerably facilitated the course of this investigation, but none is essential to the successful use of the method employed. A further analysis seems possible along the lines indicated by TISELIUS AND SANGER<sup>2</sup>, *i.e.*, by oxidation of the disulphide bridges, isolation of the four single chains and enzymatic proteolysis of each of the chains separately. These experiments must await the isolation in sufficient quantity of the four chains in chemically pure form.

#### SUMMARY

Carboxypeptidase acts on insulin, splitting off single amino acids in the first stages of the reaction. Certainly one, but possibly three of the terminal amino acids with a free carboxyl group of insulin consist of *alanine*. After 3 alanyl groups have been liberated, at least 6 amino acids appear almost or quite simultaneously.

Shortening the polypeptide chain of insulin by only a few amino acids results in the loss of the property to denature easily and in the loss of the biological activity.

#### RÉSUMÉ

La carboxypeptidase agit sur l'insuline en scindant au début de son action des acides aminés isolés. Certainement l'un, et peut-être trois, des acides terminaux de l'insuline présentant un groupe carbonyle libre, consistent en *alanine*. Après la libération de trois résidus alanyl, il apparaît, simultanément ou presque, au moins six autres acides aminés.

Un raccourcissement de quelques acides aminés seulement de la chaîne polypeptidique de l'insuline provoque la disparition de l'aptitude à la dénaturation et la perte de l'activité biologique.

#### ZUSAMMENFASSUNG

Carboxypeptidase wirkt auf Insulin indem sie in den ersten Stufen der Reaktion einzelne Aminosäuren abspaltet. Sicherlich eine, vielleicht aber auch 3 der endständigen Aminosäuren mit freier Carboxyl-Gruppe des Insulins bestehen aus Alanin. Nachdem 3 Alanylgruppen in Freiheit gesetzt worden sind, erscheinen mindestens 6 Aminosäuren gleichzeitig.

Wird die Polypeptidkette des Insulins nur um einige wenige Aminosäuren verkürzt, so verliert sie ihre leichte Denaturierbarkeit und ihre biologische Aktivität.

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## RECHERCHES SUR LE MÉTABOLISME DES PEROXYDES D'ESTERS D'ACIDES GRAS

par

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Le métabolisme des lipides peroxydés pose un double problème. Celui, d'abord, des peroxydes exogènes, apportés normalement par l'alimentation. Celui, en second lieu, des peroxydes endogènes. Car dans certains tissus, tels que les éléments figurés du sang, les endothéliums vasculaires et pulmonaires, les lipides, émulsionnés, se trouvent en milieu saturé d'oxygène et en présence de pigments hématiniques dont le rôle catalytique est bien connu. Des peroxydes existent-ils dans les tissus? Sinon, comment l'organisme en empêche-t-il l'apparition?

Ces problèmes ont été peu étudiés. Un exposé des travaux, qui s'y rapportent, a été donné par BURR ET BARNES<sup>1</sup>. GYÖRGY *et al.*<sup>2</sup>, ont montré que des rats nourris avec des régimes contenant 16 % d'acide linoléique oxydé présentent des troubles graves: chute du poids, anémie et leucopénie, troubles qui sont évités, si l'on ajoute au régime de la levure de bière. Mais, ces faits peuvent recevoir deux interprétations: ou il s'agit d'une toxicité propre aux graisses peroxydées; ou bien, il y a simplement destruction, par ces graisses, des vitamines facilement oxydables, telles que les vitamines A, E, D, l'acide linoléique et d'autres peut-être. BURR ET BARNES ont repris ces expériences et les ont continuées. Ils ont pu montrer que la levure de bière avait des propriétés antioxygènes, ce qui explique en partie son effet; et que, par ailleurs, si l'on donnait de l'huile de foie de morue aux animaux nourris avec des régimes contenant des graisses oxydées, les mêmes troubles étaient observés, bien que l'on trouve de la vitamine A dans leur foie. QUACKENBUSCH<sup>3</sup> estime que l'action pathogène des graisses rances est due à la fois à la destruction des vitamines et à une toxicité propre.

Il est intéressant de rapprocher ces faits de l'observation de CHAIX ET BAUD<sup>4</sup>, constatant que *Glaucoma piriformis* est lysé par le linoléate de sodium de façon plus active si l'acide linoléique a vieilli, que s'il est fraîchement préparé.

IRWIN, WEBER, STEENBOCK ET GODFREY<sup>5</sup> ont étudié l'absorption intestinale de graisses peroxydées. Ils ont montré que cette absorption (mesurée à partir du taux d'acides gras retrouvés dans le tube digestif) était d'autant plus lente que leur indice de peroxyde était plus grand, et, parallèlement, que leur point de fusion était plus élevé. DAM ET GRANADOS<sup>6</sup> ont montré que l'injection sous-cutanée d'huile de foie de morue légèrement peroxydée provoque localement une coloration brune et la formation de peroxydes. Cette même coloration brune de la graisse et cette peroxydation sont observées à la suite d'ingestion d'huile de foie de morue chez des poulets carencés en tocophérol.

Il résulte de cette courte revue que les connaissances concernant la destinée des peroxydes chez l'animal normal sont encore très fragmentaires. Cette ignorance s'explique par le fait que cette étude pose des problèmes techniques qu'il a été nécessaire de résoudre d'abord, et que nous commencerons par exposer.

### PARTIE EXPÉRIMENTALE

#### TECHNIQUES

##### a) *Méthode de dosage des peroxydes*

La méthode classique utilise l'oxydation de l'acide iodhydrique et le dosage iodométrique, selon la technique de LEA ou celle de WHEELER, modifiée par KING, ROSCHEN ET IRWIN<sup>7</sup>. Elle ne pouvait

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nous convenir pour deux motifs: d'une part, parce que l'iode libéré peut être en partie fixé par certaines substances biologiques; LEA<sup>8</sup> a montré que cette fixation était, dans les huiles naturelles, faible et souvent négligeable, mais en présence de certains corps comme la vitamine A à concentration élevée, il n'en est plus de même<sup>10</sup>. D'autre part, parce que c'est une macrométhode ou tout au plus une semi-microméthode, qui exige de 10 à 100 mg de lipides pour sa mise en œuvre.

La méthode de LIPS, CHAPMAN ET MAC FARLANE<sup>9</sup>, basée sur l'oxydation du fer ferreux en fer ferrique en milieu acétonique, et son dosage sous forme de sulfocyanure, prête à une autre critique: la réaction inverse est provoquée par les diphenols, les tocophérols, les caroténoides. Elle ne peut donc être utilisée sans incertitude en milieu biologique.

Nous avons étudié une méthode nouvelle<sup>10</sup> basée, comme celle de LEA, sur l'oxydation de l'acide iodhydrique. Mais l'iode libéré est immédiatement fixé par un mercaptan, la thiofluorescéine. Ce corps, bleu en milieu alcalin, est incolore sous sa forme oxydée. On termine donc par un dosage colorimétrique\*. Cette méthode est exempte des défauts de la méthode iodométrique classique: l'iode est fixé entièrement par la thiofluorescéine et les quantités de graisse mises en œuvre sont de l'ordre de 0.2 à 2 mg. Le mode opératoire est d'ailleurs très simple.

#### b) *Extraction des peroxydes des tissus*

La méthode d'extraction nous a posé des problèmes qui ont été difficiles à résoudre<sup>11</sup>. Plusieurs causes d'erreurs, inattendues, ont compliqué ce travail. Nous en exposerons sommairement la marche.

La technique d'extraction devait être a priori totale, c'est-à-dire extrayant tous les lipides tissulaires et rapide, pour éviter soit la formation de peroxydes nouveaux, soit leur polymérisation, qui se produit, en couches minces, avec une extrême facilité. Ni la méthode de KUMAGAWA, avec ses six heures d'extraction, ni la méthode de BLOOK, qui conduit à de grands volumes liquides, ne pouvaient être adoptées.

Après de nombreux essais, nous nous étions arrêtés au procédé consistant à extraire les triglycérides selon la méthode suggérée par ARTOM (agitation des tissus broyés avec de l'acétone)<sup>12</sup> et les phospholipides par l'alcool bouillant dans un appareil spécialement conçu, de telle sorte que le solvant ayant traversé le tissu broyé soit immédiatement recueilli et refroidi. L'expérience avait montré que nous obtenions ainsi la quasi-totalité des lipides, avec de petits volumes liquides et une perte de peroxydes faibles.

Appliquant alors cette technique au dosage biologique, nous n'avons retrouvé que des quantités extrêmement faibles de ces corps, même dans des circonstances telles qu'ils devaient exister en quantités considérables, par exemple, dans le tube digestif de Rats sacrifiés immédiatement après l'ingestion de peroxydes. L'explication de ce phénomène nous a été donnée par la constatation suivante: si l'on mélange des peroxydes avec un tissu broyé, ils disparaissent rapidement. Ils subsistent en grande partie si le tissu a été préalablement desséché. Nous verrons plus loin l'explication de ce fait. La conséquence en est que toute méthode de dosage des peroxydes qui ne comporte pas une dessiccation immédiate du tissu est sans valeur.

Pour ce motif, et pour d'autres qui apparaîtront dans la suite de notre exposé, nous avons adopté la technique suivante. Les tissus sont immédiatement broyés avec 10 fois leur poids de  $\text{SO}_4\text{Na}_2$  anhydre pur. On place la quantité correspondant à 1 g de tissu dans une colonne de 15 mm de diamètre intérieur, et on extrait par 40 ml d'acétone fraîchement distillée sur permanganate de potassium, puis sur un mélange de chlorure de calcium et d'hydroxyde de calcium.

\* Depuis la publication de notre mémoire, l'expérience nous a montré qu'il était préférable, lors de la préparation de la thiofluorescéine, de précipiter ce corps, non par l'acide acétique, comme nous le disions, mais par l'acide chlorhydrique dilué. Le produit obtenu est beaucoup plus stable. Il est par ailleurs inutile d'isoler le sulfhydrate de sodium par le benzène.

Cette extraction est incomplète. Mais contrairement aux indications d'ARTOM, l'acétone extrait, outre les triglycérides, environ 40 % du P. soluble dans le chloroforme<sup>12</sup>. Il paraît impossible de trouver une méthode parfaite, c'est-à-dire qui extraie tous les lipides sans détruire les peroxydes.

Une fraction connue de l'acétone est évaporée sous vide dans le petit flacon qui servira au dosage des peroxydes.

Ajoutons enfin que tous nos essais ont été faits avec de l'oléate d'éthyle préparé à partir d'acide oléique contenant moins de 1 % d'acide linoléique, et pas d'acide linoléique en quantité dosable. Il était peroxydé par exposition à l'air, en couche de 0.5 mm environ, à 100° pendant 24 heures. Les indices obtenus, de l'ordre de 400 à 500, se trouvaient toujours sur la partie ascendante de la courbe de peroxydation.

### RÉSULTATS

#### a) Les peroxydes dans le tube digestif

On introduisait par sondage dans l'estomac de Rats (jeunes adultes à jeun depuis 24 heures) une quantité de peroxydes correspondant à environ  $10000 \cdot 10^{-8}$  atm d'oxygène actif\*. Au bout d'un temps donné, l'animal était sacrifié, le tube digestif lié à ses deux bouts, prélevé en entier et extrait. Les résultats obtenus sont donnés dans le Tableau I.

TABLEAU I

Temps de digestion (heures)	Peroxydes ingérés (unités)	Peroxydes retrouvés (unités)	Fraction retrouvée %
1.5	10 400	5 900	56
3	14 100	4 250	30
3	8 000	1 900	24
5	9 500	1 950	20
5	10 600	1 250	12

Ainsi on assiste à une disparition progressive des peroxydes dans le tube digestif, en un temps correspondant à peu près à celui de la digestion des graisses peroxydées<sup>5</sup>.

#### b) Les peroxydes dans les tissus

Nous avons recherché les peroxydes dans les tissus suivants: foie, cerveau, poumons, rein, sang, dans les mêmes conditions, c'est-à-dire après administration de 10000 unités. Les animaux étaient sacrifiés 3 heures après ingestion, alors que 70 à 75 % des peroxydes avaient disparu du tube digestif. On pouvant en déceler avec certitude 5 unités par gramme de tissu, soit la 1/1500e partie de la quantité disparue. Dans un cas, nous en avons trouvé 4 unités par g dans le foie, quantité inférieure aux erreurs expérimentales. Dans tous les autres cas, nous n'avons pas pu en mettre en évidence.

Seul le tissu adipeux (graisse périrénale) présente des traces dosables de peroxydes:

Unités ingérées	Unités trouvées par g de graisse
14 000	5.2
9 000	7.5

\* Nous exprimerons désormais les peroxydes en unités, une unité étant égale à un atome-gramme d'oxygène actif  $\times 10^{-8}$ .



Ces faibles teneurs provenaient-elles des peroxydes ingérés? Pour le déterminer nous avons répété la mesure sur des animaux nourris au régime standard ("Ratigène"). On a trouvé par g de graisse 6 et 10 unités, soit des quantités du même ordre. Ainsi leur origine digestive n'est nullement démontrée.

Ces résultats sont en contradiction avec ceux que nous avons exposés dans une note préliminaire<sup>14</sup>. Dans des expériences conduites selon un mode expérimental un peu différent, nous avons retrouvé dans divers tissus des quantités notables de peroxydes. Nous avons recherché avec soin l'origine de cette divergence, en reprenant ces essais avec des modes expérimentaux variés, sans pouvoir retrouver jamais de peroxydes tissulaires. Une discussion détaillée serait trop longue pour être reproduite ici<sup>11</sup>. La conclusion en est la suivante: on sait que les corps dosés en bloc sous le nom de peroxydes sont constitués par une série d'isomères<sup>15, 16</sup>, même lorsqu'on part d'un ester pur. A fortiori, si l'on oxyde un produit complexe, doit-on obtenir un très grand nombre de composés. Dans nos essais préliminaires nous avons utilisé comme point de départ un acide oléique commercial, certainement impur. Sans exclure de façon absolue la possibilité d'une erreur technique lors de ces essais, nous pensons qu'il est possible que certains peroxydes subsistent momentanément dans les tissus après ingestion. Les conclusions du présent travail ne sont donc valables que pour l'oléate d'éthyle pur, peroxydé dans les conditions que nous avons précisées. Des études ultérieures montreront jusqu'à quel point ces conclusions peuvent être généralisées.

#### c) Destruction des peroxydes par les tissus

Nous avons dit que les peroxydes étaient détruits par les tissus broyés. Le phénomène est complexe, et nous ne l'avons pas encore élucidé entièrement. Les faits fondamentaux sont les suivants:

3 portions d'un même foie de rat, pesant chacune 2.3 g sont broyées respectivement avec 38, 31 et 35 mg de peroxydes d'indice 525. A des temps variables, les tissus sont broyés avec du sulfate de sodium anhydre et extraits par l'acétone. On dose la quantité contenant initialement 0.2 mg de peroxydes, c'est-à-dire 21 unités. Les résultats sont donnés dans le Tableau II.

TABLEAU II

Dosage après minutes	Unités retrouvées
0	21
30	14
90	10

Cette destruction des peroxydes est très diminuée si le tissu est desséché par du sulfate de sodium anhydre.

Un foie de rat est broyé avec 4 fois son poids de sable. On en fait 3 parts, correspondant chacune à 1 g de foie. La première est broyée avec 23 mg de peroxydes, puis aussitôt avec du sulfate de sodium, et extraite à l'acétone. On retrouve 99% de la quantité théorique.

La seconde reçoit 22 mg de peroxydes. Au bout d'une heure on dessèche par du sulfate de sodium et on extrait à l'acétone. On retrouve 35% de la quantité initiale.

La troisième reçoit 25 mg de peroxydes. On dessèche aussitôt par du sulfate de

sodium. On extrait et on dose au bout d'une heure: on retrouve 77% de la quantité initiale.

Si le tissu est traité deux minutes par l'eau bouillante, l'activité est conservée. Elle subsiste encore si le foie ainsi coagulé est lavé à l'eau et à l'acétone, mais il est nécessaire de restituer l'eau au tissu pour qu'il ait son activité maximum. Le cyanure de potassium ne modifie pas l'activité du tissu.

L'extraction par l'acool bouillant, sous courant gaz carbonique dans un appareil du type KUMAGAWA montre qu'une substance active est ainsi détachée des protéines par l'alcool. Mais cette libération est très longue:

0.5 g de foie sont coagulés par l'eau bouillante, broyés avec 5 g de sable, lavés 3 fois à l'eau et 3 fois à l'acétone. On extrait ensuite sous gaz carbonique par l'alcool bouillant soit pendant 30 minutes, soit pendant 3 heures. Les résultats sont donnés dans le Tableau III.

TABLEAU III

Fraction	Peroxydes disparus % après traitement de	
	30 minutes	3 heures
Extrait alcoolique .	87	91
Résidu . . . . .	82.5	46
Témoin . . . . .	4	

Ainsi, après 3 heures d'extraction, une notable partie du produit actif est encore liée aux protéines. La substance, ainsi extraite est active, même en l'absence d'eau; mais celle-ci en accroît nettement l'action. Enfin, si l'extrait alcoolique est évaporé, repris par un petit volume de chloroforme et traité par un grand volume d'acétone, la substance se partage entre le liquide et le précipité phospholipidique. Des précipitations répétées accumulent dans l'acétone la plus grande partie de l'activité.

Tels sont les faits expérimentaux certains, dont l'interprétation est relativement simple. D'autres sont encore mal coordonnés; il est possible qu'il existe plusieurs substances actives. Il est remarquable, en particulier, que si l'on utilise de faibles quantités de peroxydes (de l'ordre de 2 mg par g de tissu), la destruction se fasse de façon extrêmement rapide, sans que la dessiccation du tissu la modifie. D'autre part, nous avons obtenu des extraits aqueux actifs, sans que nous puissions déterminer s'il s'agit d'une substance particulière ou d'une simple dissolution de protéines incomplètement coagulées. Ces points devront être examinés ultérieurement.

Le foie n'est certainement pas le seul organe qui contienne ces corps. Il résulte de quelques essais qu'ils semblent répandus dans tous les tissus. Le sérum sanguin, en particulier, est remarquablement actif: 100 ml de sérum étant agités avec 100 mg de peroxydes, on retrouve 16% seulement de ceux-ci après 10 minutes.

#### DISCUSSION

Si l'on s'en tient aux faits acquis, ils tendent à démontrer l'existence d'une substance au moins détruisant les peroxydes lipidiques avec une rapidité très grande. De travaux exécutés dans ce laboratoire<sup>17</sup>, il semble résulter que pour qu'une substance soit

antioxygène, il faut: a) qu'elle détruise les peroxydes; b) qu'elle résiste à l'autoxydation. *In vivo*, la substance inconnue se présente à cet égard comme un antioxygène puissant. Autant qu'on puisse l'affirmer avant isolement, elle détruit les peroxydes au moins cent fois plus vite que le tocophérol, et elle est stable. *In vitro*, il n'en est peut-être pas de même, car, libérée des protéines, elle paraît très fragile.

Il est vraisemblable que c'est à ces substances qu'il faut attribuer l'absence de peroxydes dans les tissus après ingestion. Il est évidemment possible qu'ils soient détruits dans le tube digestif. Mais il nous paraît plus probable qu'ils traversent la barrière intestinale, et qu'ils disparaissent sous l'action de ces corps inconnus. Sans doute est-ce à eux aussi qu'il faut rapporter la longue conservation de la vitamine A dans les foies conservés humides, alors qu'elle est rapidement détruite, quand ils sont desséchés.

### RÉSUMÉ

On s'est proposé de chercher, ce que deviennent les peroxydes d'esters gras, après ingestion chez le rat. On utilisait l'oléate d'éthyle pur, d'indice voisin de 500.

La méthode d'extraction comportait une dessiccation du tissu par le sulfate de sodium anhydre, suivie d'une extraction par l'acétone. On montre, qu'aucune autre méthode ne paraît utilisable actuellement. Les dosages de peroxydes étaient pratiqués à l'aide de la thiofluorescéine.

Les peroxydes disparaissent progressivement du tube digestif: il en reste environ 15 % après 5 heures.

On n'en retrouve pas dans les tissus en quantités dosables (supérieures à  $5 \cdot 10^{-8}$  atome-gramme d'oxygène actif par g). Seul le tissu adipeux paraît en contenir de faibles quantités, dont l'origine digestive n'est pas démontrée.

Les tissus détruisent très rapidement les peroxydes, selon un mécanisme, peut-être complexe. Une substance active au moins est thermostable et liée aux protéines. Elle en est détachée par une longue extraction à l'alcool bouillant. Elle est soluble dans l'acétone.

L'activité des tissus est très diminuée lorsqu'ils sont desséchés. Il faut, sans doute, attribuer à ce fait la conservation de la vitamine A, dans les foies conservés humides, et sa rapide disparition lorsqu'ils sont desséchés.

### SUMMARY

The fate of the peroxides of fatty acid esters after ingestion in the rat has been investigated. Pure ethyl oleate (P.V. about 500) was used.

The method of extraction includes a dehydration of the tissue with anhydrous sodium sulphate, followed by extraction with acetone. It is shown that no other method appears to be satisfactory. The peroxide determinations were made by the thiofluorescein method.

The peroxides are gradually eliminated from the digestive tract; at the end of 5 hours about 15 % remains. No determinable quantities (greater than  $5 \cdot 10^{-8}$  gram atom of active O per g) have been found in the tissues. Adipose tissue alone appears to contain small quantities, the digestive origin of which is not proved.

The tissues destroy peroxides very rapidly, through a mechanism which may be complex. At least one active substance is thermostable and combined with proteins. It is separated from the latter on prolonged extraction with boiling alcohol and is soluble in acetone.

The activity of the tissues when dehydrated is very much decreased. The stability of vitamin A in moist stored livers and its rapid disappearance on drying must be attributed to this fact.

### ZUSAMMENFASSUNG

Das Vorkommen von Peroxyden in Fettsäureestern nach Verfütterung an Ratten wurde untersucht. Wir verwendeten reines Äthyloléat (ungefähr 500).

Die Extraktionsmethode bestand aus Trocknen der Gewebe gefolgt von Ausziehen mit Aceton. Nach 5 Stunden blieben noch 15 % übrig. Es konnte keine messbare Menge (grösser als  $5 \cdot 10^{-8}$  Atom-gramm aktiven Sauerstoffs pro g) gefunden werden. Nur das Fettgewebe scheint geringe Mengen davon zu enthalten, es ist aber nicht bewiesen, dass sie von der Verdauung herrühren.

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Durch Gewebe werden die Peroxyde schnell nach einem vielleicht komplexen Reaktionsmechanismus zerstört. Wenigstens eine aktive Substanz ist hitzebeständig und an Eiweiss gebunden. Durch Ausziehen mit kochendem Alkohol wird diese Substanz in Freiheit gesetzt. Sie ist auch in Aceton löslich. Durch Trocknen wird die Aktivität der Gewebe stark herabgesetzt.

Vielleicht hängt die Haltbarkeit von Vitamin A in feuchten konservierten Lebern und seine rasche Zerstörung beim Trocknen mit dieser Tatsache zusammen.

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## CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF THE FLAGELLA OF *PROTEUS VULGARIS* AND *BACILLUS SUBTILIS* A COMPARISON

by

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The preparation of flagella from *Proteus vulgaris* in a highly purified state has been previously described<sup>1</sup>. In order to find out possible differences in the structures of flagella from different species, the same scheme of preparation has now been applied to a strain of *Bacillus subtilis*.

As has been already mentioned<sup>1</sup>, the flagella are reversibly precipitable with ammonium sulphate. Further chemical and serological tests, described below, have not shown it probable that any damaging effect of this salt precipitation is likely. In addition, sulphur analyses have not revealed that any appreciable amount of sulphate is absorbed during the precipitation. Therefore, in order to remove possible impurities, a repeated precipitation with ammonium sulphate to half saturation has been inserted in the preparation scheme after the first two centrifugations at 27 000 r.p.m. (*c.f.*<sup>1</sup>). The sulphate was brought to p<sub>H</sub> 7 with ammonia. As will be shown below, the precipitation has a small but distinct purification effect on the flagellar preparations, and the procedure is now applied in the routine preparations. The analytical figures in this paper refer, unless otherwise mentioned, to salt precipitated flagella.

On the flagella from the two species analyses for nitrogen, phosphorus, amino acids, carbohydrate and fatty material have been performed. Moreover, some serological and physicochemical observations have been made.

### EXPERIMENTAL

The methods for determining dry weight, nitrogen and phosphorus have been described previously<sup>1</sup>. The nitrogen content of the salt precipitated *Proteus* flagella have been determined to 16.3–16.5%, which with regard to the used micro-methods can be regarded as a variation within the experimental errors. The value found is somewhat higher and more constant than for non-salt-precipitated flagella. This difference will be discussed later in the present paper in connection with other analytical results.

The nitrogen content of the *Subtilis* flagella was determined to 15.8–16.0%.

Only traces of phosphorous have been found in the *Proteus* flagella<sup>1</sup>. The same was true for *Subtilis* preparations.

In order to estimate carbohydrate in a semi-quantitative way the MOLISCH test

has been used in the following way: To 1 ml of the sample or of a known sugar solution was added 0.1 ml, 1%  $\alpha$ -naphthol in 50% ethanol. 1 ml conc. sulphuric acid was run under the fluid, and the solution was gently mixed. In this way 1  $\mu$ g of carbohydrate was detectable. The colour given by about 1 mg of protein in the test was compared with that of freshly prepared solutions containing 1–20  $\mu$ g saccharose.

The carbohydrate content of the *Proteus* flagella was found to be less than 0.2% whereas the *Subtilis* flagella contained 1–2% carbohydrate.

The estimation of fatty material has followed the micro-method given by BLOOR<sup>2</sup>, using ether-ethanol and petroleum ether as extracting agents. Carefully distilled solvents were used and the blank values obtained from the evaporation of the pure solvents were determined. Both *Subtilis* and *Proteus* flagella gave only 0.7–0.8% extractable material. As less than 0.1 mg of this material was obtained, it was impossible to judge its true nature.

The qualitative amino acid composition of the flagella was determined according to the paper chromatography method<sup>3</sup>. The Swedish filter paper Munktell OB was used with phenol-collidine and n-butanol-collidine as developing agents for the two-dimensional chromatograms. A few  $\mu$ g of individual amino acids, except histidine, were easily detectable in artificial mixtures after spraying with 0.2% ninhydrin in butanol and drying at 100°. Histidine sometimes entirely disappeared in the chromatograms or gave only weak spots. This has also been found to be true by other investigators<sup>4</sup>.

In the phenol-collidine diagrams all amino acids were separated with the exception of methionine and the leucines. In the butanol-collidine diagrams, however, the methionine forms a quite distinct spot. The leucines were not separated altogether here, but enough for detecting the separate components in a mixture. The other amino acids except the basic and dicarboxylic ones formed distinct spots in the butanol diagrams and therefore the two types of chromatograms could be checked against each others.

The flagellar preparations were hydrolysed with 6-N HCl for 24 hours at 105°. After completed hydrolysis the acid and water was evaporated over soda lime and sulphuric acid. 100–500  $\mu$ g of the hydrolysate were applied on the filter paper, ammonia vapour was allowed to act on the dry spot for a short time and the chromatogram was made.

In the *Proteus* diagrams strong spots were obtained indicating the following amino acids: Arginine, lysine, aspartic and glutamic acid, glycine, serine, alanine, threonine, valine, leucine, isoleucine, phenylalanine and tyrosine (a somewhat weaker spot). A very weak but constantly appearing methionine spot was obtained. With 500  $\mu$ g of amino acids a somewhat doubtful proline spot also appeared. Histidine, tryptophane, cystine, hydroxyproline and norleucine could not be detected in the diagrams, indicating in any case a very low content of these acids. Special, still more sensitive tests for these acids (except norleucine) were applied (see below). The *Subtilis* diagrams appeared quite the same, except for a stronger methionine spot and a weak but constantly appearing proline spot.

No spots of unknown origin were obtained in the diagrams.

#### *Determination of the separate amino acids*

The spectrophotometric method of determining tyrosine and tryptophane, according to GOODWIN AND MORTON, has already been referred to <sup>1, 5</sup>. As has already been mentioned, no tryptophane could be detected in the *Proteus* flagella, and the same case was found to be true for the *Subtilis* preparations. In this connection it may also be

mentioned that no absorption, indicating nucleic acids, has been found in these preparations.

The results of the tyrosine determinations are given in Table I.

TABLE I

Prep.	% tyrosine-N/total N
Proteus I	0.842
„ II	0.813
„ III	0.847
Subtilis I	0.569
„ II	0.586

*Cystine and cysteine.* The phosphotungstic acid method according to SCHÖBERL AND RAMBACHER<sup>6</sup> was used. Less than 0.05% (limit sensitivity of the method) was found to be present in both *Proteus* and *Subtilis* flagella.

*Hydroxyproline.* The method of MCFARLANE AND GUEST<sup>7</sup> was employed. Less than 0.1% was present in the preparations investigated.

*Histidine.* The Sakaguchi test performed according to MCPHERSON<sup>8</sup> showed less than 0.2% histidine in the flagella (a yellow colour appearing in the test made it less sensitive than expected).

*Methionine.* The colorimetric method of MCCARTHY AND SULLIVAN<sup>9</sup> was employed. The *Proteus* flagella showed a low but constant methionine content of 0.3%, whereas the *Subtilis* preparations gave values between 0.4–1%.

#### Serological tests

Only *Proteus* preparations have been investigated so far. In order to get rabbit antisera, 0.5–1 mg of the flagella were inoculated four times every five days. Five days after the last inoculation, heart puncture was performed.

The flagella gave antisera, which agglutinated the corresponding bacterial suspension to as high a titer as an antiserum, produced by the bacterial suspension itself. The flagella were precipitated by the corresponding H-antiserum.

#### DISCUSSION

*The question of purity.* As for *Proteus*, the precipitation with ammonium sulphate has resulted in a somewhat higher and more reproducible N-content than has been reported previously<sup>1</sup>. Moreover the carbohydrate content has diminished from 1% to less than 0.2%. Also, after disintegrating the flagella at a  $p_H$  of 3 and centrifuging the solution at 27000 r.p.m. for 60 minutes a deposit was obtained that after washing amounted to only about 1% of the dry weight of the flagella. For preparations, not precipitated with ammonium sulphate, a value of about 5% was obtained<sup>1</sup>. Moreover since the chemical analyses referred to above have given reproducible values, it seems justified to regard the preparations of the *Proteus* flagella, especially after salt precipitation, as of a high degree of purity. At the lowest estimate more than 95% of flagellar material can be judged to be present in the preparations.

The preparations of *Subtilis* flagella are without doubt less pure. Thus, these preparations, when centrifuged down, have in most instances been light brown coloured to a small extent. The deposit after centrifuging the disintegrated flagella has amounted to about 3%. The somewhat lower nitrogen content and higher carbohydrate content of these flagella in comparison with *Proteus* is most probably due to impurities, since less pure *Subtilis* preparations (cultivated at higher temperatures) have shown an even lower N-content.

*Comparison between Subtilis and Proteus flagella.* Both flagella show the property of being disintegrated at low  $p_H$ -values (3-4) into smaller particles with a sedimentation constant of 2-2.5 S. The same also holds for flagella from *Salmonella paratyphi*<sup>10</sup>; so this seems to be a common property for bacterial flagella from different species indicating a close structural resemblance.

The low content of carbohydrate and of possibly fatty material (probably impurities of varying amounts), in the different flagella, is noticeable, especially in connection with the antigenic properties of the flagella.

The flagella have been shown to be of protein nature. The qualitative analysis of the different amino acids has given almost the same result for *Proteus* and *Subtilis* flagella (proline is possibly missing in the former). Some amino acids as cystine (and cysteine), tryptophane, histidine and hydroxyproline present in most of other proteins are missing altogether in the flagella investigated, indicating a close relationship between the flagellar proteins of the different species. However, reproducible quantitative differences seem to exist as has been shown by the tyrosine analysis.

The author is very much indebted to Professors ARNE TISELIUS and SVEN GARD for valuable discussions and criticism. The author also wishes to express his gratitude to Professor THE SVEDBERG for the privilege of working at the Institute of Physical Chemistry.

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#### SUMMARY

Chemical analyses, physicochemical and serological tests have been performed on flagella, prepared in a highly purified state from cultures of *Proteus vulgaris* and *Bacillus subtilis*.

Like *Salmonella paratyphi* flagella, the flagella investigated here are disintegrated at  $p_H$  3 into smaller units with a sedimentation constant of 2-2.5 S.

15.9-16.4 % nitrogen is found in the flagella, but no phosphorus or nucleic acids.

Only a low content of carbohydrate and possibly fatty material is found, both probably impurities.

The flagella are of protein nature and the following amino acids are found: arginine, lysine, aspartic and glutamic acid, glycine, serine, alanine, threonine, valine, methionine, leucine, isoleucine, phenylalanine and tyrosine. Tryptophane, histidine, cystine + cysteine, and hydroxyproline have not been found to be present. The analyses have shown the same qualitative amino acid composition for the different flagella. However, certain quantitative differences exist.

#### RÉSUMÉ

Des analyses chimiques et des essais physicochimiques et sérologiques ont été faits sur des flagelles préparés à un haut degré de pureté, et provenant de cultures de *Proteus vulgaris* et *Bacillus subtilis*.

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Comme les flagelles de *Salmonella paratyphi*, ceux qui font l'objet des présentes recherches sont désintégrés à pH 3 en fragments plus petits, présentant une constante de sédimentation de 2 à 2.5 S.

Les flagelles contiennent de 15.9 à 16.4 % d'azote, mais ni phosphore ni acides nucléiques. On n'y trouve que de faibles quantités d'hydrates de carbone et de matières grasses qui ne sont probablement que des impuretés.

Les flagelles sont de nature protéique; on y trouve les acides aminés suivants: arginine, lysine, acides aspartique et glutamique, glycocholate, sérine, alanine, thréonine, valine, méthionine, leucine, isoleucine, phénylalanine et tyrosine. Semblent manquer: tryptophane, histidine, cystine + cystéine et hydroxyproline. Les analyses montrent que les différents flagelles ont la même composition qualitative, mais il existe des différences quantitatives.

### ZUSAMMENFASSUNG

Geisseln, welche in weitgehend gereinigtem Zustand aus Kulturen von *Proteus vulgaris* und *Bacillus subtilis* hergestellt worden waren, wurden der chemischen Analyse sowie physikochemischen und serologischen Prüfungen unterworfen.

Wie die Geisseln von *Salmonella paratyphi*, so zerfallen auch die hier untersuchten Geisseln bei pH 3 in kleinere Einheiten mit einer Sedimentationsgeschwindigkeit von 2–2.5 S.

In den Geisseln wurde ein Stickstoffgehalt von 15.9–16.4 % gefunden, aber kein Phosphor und keine Nukleinsäuren. Ein niedriger Gehalt an Kohlenhydraten und vielleicht an Fetten, muss wahrscheinlich auf Verunreinigungen zurückgeführt werden.

Die Geisseln haben Eiweisscharakter und die folgenden Aminosäuren wurden darin festgestellt: Arginin, Methionin, Lysin, Asparagin- und Glutaminsäure, Glycin, Serin, Alanin, Threonin, Valin, Methionin, Leucin, Isoleucin, Phenylalanine und Tyrosin; Tryptophan, Histidin, Cystin-Cystein und Hydroxyprolin wurden nicht gefunden. Die Analysen haben für verschiedene Geisseln qualitativ dieselbe Zusammensetzung an Aminosäuren ergeben. Dagegen gibt es gewisse quantitative Unterschiede.

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STUDIES ON THE AUTOLYTIC SYSTEMS OF GRAM POSITIVE  
MICRO-ORGANISMSI. THE LYTIC SYSTEM OF *STAPHYLOCOCCI*

by

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## INTRODUCTION

The phenomenon of bacterial autolysis is well known and has been the subject of numerous investigations since the early observations of RETTGER (1904), but only infrequent attempts have been made to study the chemical reactions concerned in the process. Of these, detailed studies have been chiefly concerned with the pneumococcus. In the preliminary investigations with this organism, crude preparations of an intracellular protease and a lipase, as well as several carbohydrate-splitting enzymes were isolated by AVERY AND CULLEN (1920a, 1920b, 1920c) from autolysed broth cultures. These enzymes possessed several features in common, *e.g.*, they were absent from the culture medium during the early phase of active growth, were easily denatured by heat, had  $p_H$  optima 7.0–7.8, and were inactive at  $p_H$  4.5. Following this it was shown (AVERY AND CULLEN, 1923) that autolysates containing the cellular substances of the pneumococci possessed an enzyme, or a group of enzymes, capable of exerting a lytic action on suspensions of heat-killed pneumococci and, to a less extent, on suspensions of the closely related *Streptococcus viridans*. The enzyme corresponded closely in  $p_H$  optimum and stability to the enzymes previously studied. It was not type specific in its action and had no action on *Staphylococcus aureus*.

Extension of these studies (GOEBEL AND AVERY, 1929) showed that autolysis of pneumococci was accompanied by a proteolysis resulting in an increase in amino and non-coagulable nitrogen and by a lipolysis which gave rise to the liberation of ether-soluble fatty acids. When autolysates containing the active intracellular enzymes were added to heat-killed pneumococci, lysis of the cells occurred and there was an increase in the non-coagulable nitrogen and amino-nitrogen comparable to the changes accompanying spontaneous autolysis. When incubated with emulsions of the alcohol-soluble lipoids of pneumococci, these active autolysates caused an increase in the ether-soluble fatty acids.

Confirmation of much of the previous work was provided by DUBOS (1937) who also made the significant observation that, before lysis of the cells occurred, the cocci first became gram negative and this change in staining properties was not necessarily associated with actual complete lysis of the cells. The importance of this observation

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was emphasized when it was shown (THOMPSON AND DUBOS, 1938) that ribonucleic acid and a nucleoprotein were present among the substances released into solution when a suspension of rough Type II *Pneumococci* was incubated at 37° until the cells became gram negative. It was also observed that by a similar process of controlled autolysis, it was possible to release from staphylococci a substance which gave the reactions of ribonucleic acid.

Recent work by HENRY AND STACEY (1946) has revealed that gram positive cells contain at the cell surface a complex which consists of carbohydrate and the magnesium salt of ribonucleic acid, the latter being linked to basic proteins of the cell "cytoskeleton". When these components were removed from the cells by extraction at 60° with 2% sodium cholate solution, the gram staining reaction became negative. The importance of ribonucleic acid in the gram complex was shown by the fact that the residual gram negative "cytoskeleton" could, after suitable reduction, be recombined with magnesium ribonucleate to restore the initial gram positive staining characteristics.

That the ribonucleic acid and carbohydrate of the gram complex are in combination has been shown (WEBB, 1948) by the fact that hydrolysis of certain carbohydrate linkages located at or near the surface of gram positive organisms by the action of lysozyme, is accompanied by the liberation of ribonucleic acid from the cell together with a change in the staining reaction from gram positive to gram negative.

In view of these observations and following suggestions by Dr H. HENRY, it occurred to us that a study of such enzyme reactions may give rise to a more complete picture of the components of the bacterial cell. In particular, it was considered that by a detailed study of autolysis and the characterization of the enzymes involved in the various stages of cellular disintegration, a more complete understanding of bacterial structure could be obtained.

In preliminary investigations with a strain of *Clostridium welchii* and *Staphylococcus citreus* (B 9) it has been shown (Dr H. HENRY, private communication; WEBB, PH. D. Thesis, University of Birmingham, 1946) that the autolytic enzyme systems of these organisms are of similar nature and are composed of several separate and distinct enzymes. A detailed study of the change in the gram staining reaction which occurs as the first stage in the disintegration of the cells revealed that this is brought about by an enzyme which has the property of hydrolysing ribonucleic acid but not deoxyribonucleic acid. This enzyme differs from the ribonucleinase of animal tissues in several respects. It is inactive under oxidising conditions, but reactivated by reducing agents; has  $p_H$  optimum at  $p_H$  8.0 and is irreversibly inactivated at  $p_H$  4.0. At 80° it is completely inactivated, but at 60° the degree of inactivation is dependent upon the  $p_H$  of the solution, the enzyme being most stable at  $p_H$  8. Although these properties are possessed by the enzymes of both *Cl. welchii* and *Staph. citreus* they are species and, to some extent, strain specific in their action on heat-killed gram positive cells. Thus, the enzyme from *Cl. welchii* readily converts heat-killed gram positive, *Cl. welchii* to gram negative forms, but has no action on heat-killed, gram positive *Staph. citreus*, *Staph. aureus*, or *Lactobacillus helveticus*. Similarly, the enzyme from *Staph. citreus* exhibited no action against killed gram positive *Cl. welchii* or *Staph. aureus*, but was active against a rough variant isolated from an aged culture of a strain of *Staph. aureus*. In view of the latter observation it was suggested that the specificity of these bacterial enzymes was, in some way, determined by the specific carbohydrates of the cell. It was further suggested that the second stage of autolysis, namely the lysis of the gram negative cell bodies which remain

intact after the action of the nucleinase, was brought about by a proteolytic enzyme.

In the present work, the studies on the autolytic enzymes of staphylococci have been extended to the enzyme system responsible for this second stage of autolysis. The lytic enzyme system has been separated, concentrated in some degree and partially fractionated to reveal the presence of two proteolytic enzymes.

#### *Isolation of Autolytic Strains of Staphylococci*

*Staphylococcus citreus* B. 9 had been used in the studies of the first stage of autolysis and was known to autolyse with a maximum of 80–90% lysis at  $pH$  7–8 within 6 days at  $37^\circ$ . Other strains of staphylococci were isolated in pure culture by plating out pathological specimens. The autolytic properties of such strains were determined as follows:

The cells from 48 hour cultures of the organisms on glucose-peptone-agar were removed with distilled water and the resulting homogeneous suspension suitably diluted such that the addition of 10 drops from a uniform pipette to distilled water (5 ml) gave an opacity corresponding approximately to No. 10 on MACFARLAND's standard barium sulphate opacity scale. 10 Drops of this suspension were then added to each of a series of uniform test tubes containing 1.0 ml of the appropriate buffer ( $pH$  4.0–10.0 in intervals of 0.5) in physiological saline (4.0 ml). At the same time, a standard opacity scale was prepared by adding 1 to 10 drops of the cell suspension to 10 uniform tubes each containing 5 ml 2% formol saline (20 ml 40% formaldehyde in 1000 ml 0.85% sodium chloride), for it was found that direct comparison of the tubes of the experimental series with such a scale gave more accurate and reproducible results than when the barium sulphate opacity scale was used.

After the addition of toluene (0.1 ml), the tubes of the experimental series were tightly corked and incubated at  $37^\circ$ . When there occurred no further increase in the percentage of gram negative cells, as determined by the examination of stained smears (the change requiring a period which varied from 36 hours to 5 days according to the strain of staphylococcus), the corks were removed and the tubes replaced in the incubator for a further 24 hours in order to remove completely the toluene. The percentage lysis at each  $pH$  value was then determined by direct comparison of the experimental series with the standard opacity scale.

Of the organisms examined, a strain of *Staph. albus* (9238) was selected which underwent maximum autolysis (90% lysis) at  $pH$  7–8 within 48 hours (Fig. 1) with the liberation of a very active lytic enzyme system. It differed in autolytic properties from *Staph. citreus* (B 9) previously employed in that it autolysed more rapidly (48 hours compared with 5 days for the latter organism). In consequence the division of autolysis into two separate and distinct stages, namely the conversion of the gram positive cell to the gram negative state and the subsequent lysis of the gram negative cell bodies, was not so sharply defined.

#### *Proteolytic Changes during Autolysis*

It appeared that much information concerning the mode of action of the proteolytic enzymes of the autolytic enzyme system of gram positive micro-organisms was to be obtained by examination of the type (total, non-coagulable and amino) and amount of protein and peptide nitrogen liberated from the cells at intervals during autolysis.

In such an examination carried out during the autolysis of *Staph. citreus* B 9 the total nitrogen

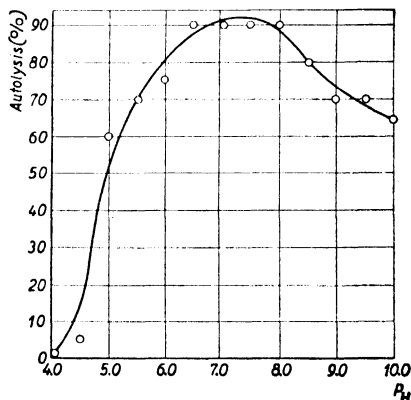


Fig. 1. Autolysis —  $pH$  curve.  
*Staph. albus* (9238)

was determined by means of a micro-Kjeldahl method capable of estimating 0.001–0.1 mg of nitrogen. The preliminary digestion was carried out in the usual manner with concentrated sulphuric acid, potassium bisulphate, copper sulphate and a trace of selenium as catalyst. As the potassium bisulphate and copper sulphate contained small quantities of nitrogenous matter, these substances were added in the form of a solution containing 120 mg of potassium bisulphate and 10 mg of copper sulphate per ml; 0.5 ml of this solution were used for each digestion. The nitrogen converted into ammonium sulphate was determined by distillation of the ammonia, in the apparatus described by MARKHAM (1942), into boric acid solution. The ammonium borate was then titrated with N/100. hydrochloric acid. Non-coagulable nitrogen was determined by adding 7 ml of 10% trichloroacetic to the required volume of solution diluted to 10 ml. After 1 hour the solution was filtered and an aliquot portion of the filtrate then analysed for total nitrogen by the micro-Kjeldahl method.

Amino nitrogen was estimated by the copper method of POPE AND STEVENS (1939).

It was realized, however, that the proteolytic enzymes were not the only enzymes present during autolysis which were capable of liberating nitrogen in these three forms. For example, the action of the ribonucleinase on the gram complex would result in the formation of soluble nitrogenous products, and these would be estimated in the solution. However, as has been shown in preliminary investigations, the proteolytic enzymes of the autolytic system are inhibited by reducing agents whereas the enzyme responsible for the conversion of the gram positive cell to the gram negative form is active under these conditions. Hence, by a comparison of the liberation of soluble nitrogen compounds from identical cell suspensions, one of which contained a suitable reducing agent, it was possible to obtain a measure of the proteolytic changes which accompany autolysis.

It was established that the estimation of total, non-coagulable and amino nitrogen was not affected by the presence of the reducing agent (hydrogen sulphide).

The experimental determinations were carried out as follows:

A suspension of the washed cells from 20 Roux bottles of a 48 hour culture of *Staph. citreus* B 9 on glucose-peptone agar in distilled water (250 ml) and 0.2 M phosphate buffer pH 7.5 (310 ml) was shaken homogeneous and then divided into two equal fractions. Of these, one was made up to 350 ml with distilled water, and the other adjusted to 350 ml with a saturated aqueous solution of hydrogen sulphide. Toluene (1 ml) was added to each, and the suspensions allowed to autolyse in closed vessels at 37°. Fractions of each suspension were withdrawn at suitable time intervals, centrifuged at high speed until the supernatants were completely clear, and aliquot fractions of the solutions analysed in duplicate for total, non-coagulable and amino nitrogen. The volume of the autolysate taken for analysis was decreased during the experiment from 70 ml to 10 ml.

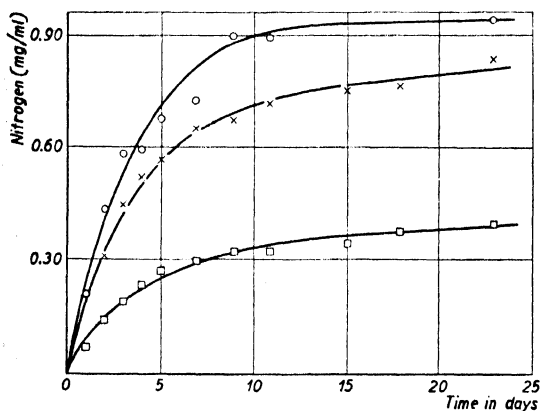


Fig. 2. Nitrogen liberated during autolysis of *Staph. citreus* (B 9). ○—○ total nitrogen, ×—× non-coagulable nitrogen, □—□ amino nitrogen

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The results obtained with the two suspensions were similar, but, in the presence of the reducing agent, the nitrogen liberated in the above three forms (Fig. 3) was considerably less than that liberated from the cells undergoing normal autolysis (Fig. 2). A measure of the total, non-coagulable and amino nitrogen liberated from the cells by the action of the enzyme inhibited by hydrogen sulphide was provided by the difference between the results of Fig. 2 and Fig. 3 and is shown in Fig. 4.

The examination of stained smears during the course of the determinations showed that in both suspensions the cells became completely gram negative after 5 days. At the end of this period, no further

lysis of the cells occurred in the suspension containing the reducing agent. In the second suspension, disintegration of the cells, as evidenced by the appearance of gram negative cell debris in the stained smears and by the clearing of the suspension, occurred. The results of Fig. 4, which show that little total nitrogen was liberated from the cells by the action of the lytic enzyme (*i.e.*, the enzyme inhibited by the reducing agent) during the first 3-4 days of the experiment, would indicate that this enzyme does not play any part in autolysis until the cells have become gram negative. Furthermore, the liberation of non-coagulable nitrogen was not appreciable until 4-5 days after the commencement of autolysis. The liberation of total nitrogen by the action of the enzyme inhibited with hydrogen sulphide is not in itself proof that this enzyme is a proteinase (endopeptidase). However, as the total nitrogen liberated during the first 8 days was mainly insoluble in trichloroacetic acid it is considered that this could only result from one type of proteolytic enzyme, namely a proteinase.

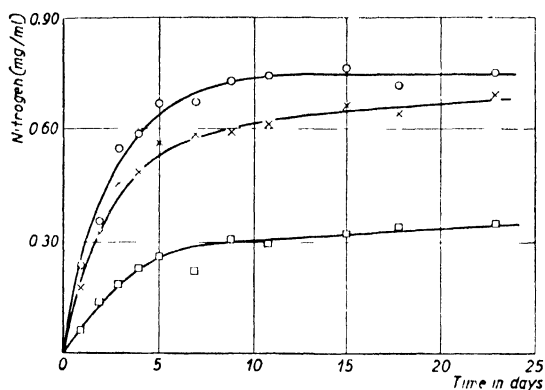


Fig. 3. Nitrogen liberated during autolysis of *Staph. citreus* (B 9) in the presence of hydrogen sulphide. o—o total nitrogen, x—x non-coagulable nitrogen, □—□ amino nitrogen

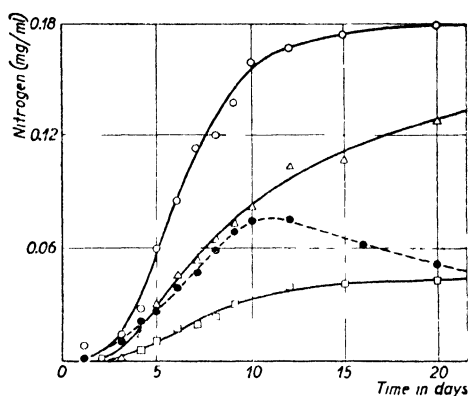


Fig. 4. Nitrogen liberated from autolysing *Staph. citreus* (B 9) by the action of the enzymes inhibited by hydrogen sulphide. o—o total nitrogen, Δ—Δ non-coagulable nitrogen, ●—● coagulable nitrogen, □—□ amino nitrogen

Fig. 4 shows that the non-coagulable nitrogen liberated continued to rise after the total nitrogen had reached a maximum value. The liberated coagulable nitrogen attained appreciable values after 3-4 days autolysis, reached a maximum after 8-10 days, and then decreased. From these results it was concluded that protein material was first liberated from the autolysing cells in a form insoluble in trichloroacetic acid solution and then underwent subsequent enzymatic hydrolysis into smaller units soluble in trichloroacetic acid. The fact that the amino nitrogen was but slowly increasing during this period showed that these units were polypeptides of relatively high molecular weight and were, therefore, produced by the hydrolysis of peptide bonds in the middle of polypeptide chains. That is, by the action of a proteinase (endopeptidase).

That the suspension of gram negative cells in which lysis had been inhibited by the presence of the reducing agent, still contain an active lytic enzyme was shown as follows:

The washed centrifuged suspension was suspended in 0.05 M citrate buffer pH 5.0 and a current of air drawn through for 6 hours to remove the last traces of hydrogen sulphide and to ensure oxidising conditions. The suspension was then centrifuged and the cells washed twice with distilled

water. 10 Drops of a suspension of the cells in distilled water were added from a pipette to each of a series of uniform test-tubes containing physiological saline (4.0 ml) and 1.0 ml of the appropriate buffer (pH range 4.0–10.0). The series was incubated at 37° for 24 hours and the degree of lysis in each tube then determined by comparison with a standard opacity scale prepared from the gram negative cells.

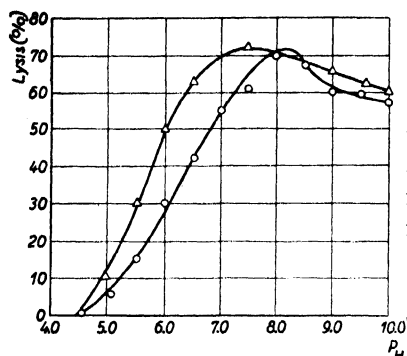


Fig. 5. pH optimum for the lysis of the gram negative cytoskelctons of *Staph. citreus* (B 9) by the lytic enzyme of the autolytic system (o—o) and by trypsin (Δ—Δ)

Control solutions containing enzyme preparations which had been heated at 80° for 30 minutes showed that no autohydrolysis of the substrates occurred under the conditions of the experiments and that the proteolytic enzymes were deactivated at 80°. Further, no appreciable self-hydrolysis was observed when the enzyme solutions were incubated alone.

The accuracy of the formol titration in the determination of proteinase action has been questioned. NORTHROP AND KUNITZ (1932) considered that the method was not a direct measure of the initial hydrolysis of the protein, but only determined the changes which occurred after the latter had been partially hydrolysed by the proteinase. However, in the present work it was considered that the use of NORTHROP'S modification of the formol titration was justified for it was found that using 5 ml quantities of solution titrated with 0.01 N sodium hydroxide, 1% of the maximum attainable hydrolysis of casein could be easily measured. Thus, it was possible to detect the hydrolysis of one peptide bond in every hundred. Hydrolysis of this order would result in the production of polypeptides containing on average 100 amino acid residues and of average molecular weight, 11500. The hydrolysis of the casein substrate produced by the enzyme from *Staph. citreus* (B 9) never exceeded 5% of the value obtained by acid hydrolysis according to the method of VAN SLYKE (1912) and which therefore, represented the production of polypeptides of an average molecular weight of 2300. That the production of polypeptides of such molecular weight is due almost entirely to the action of a proteinase is evident from the work of WINNICK (1944) in which it was shown that the products remaining after the action of crystalline proteolytic enzymes on casein ranged from pentapeptides (molecular weight about 600) to heptapeptides (molecular weight about 800).

#### *The presence of proteolytic enzyme in Staphylococcus autolysates*

a) *Staph. citreus* (B 9). The experiments already described indicate that the proteolytic enzymes of the autolytic system do not come into operation until most of the cells have become gram negative. Therefore, in order to obtain an enzyme preparation relatively free from the enzyme responsible for the conversion of the gram positive

cell to the gram negative state, autolysis was allowed to proceed at  $p_H$  7 until the cells were gram negative. The suspension was then centrifuged, the cells resuspended at  $p_H$  8 and allowed to autolyse further until examination of stained smears revealed marked disintegration of the gram negative cells.

The experimental procedure was as follows:

Cells from 10 Roux bottles of a 48 hour culture of *Staph. citreus* B 9 on EVANS' peptone-glucose-agar were suspended in water (80 ml) and 0.2 M phosphate buffer  $p_H$  7.0 (40 ml). Toluene (6 ml) was added and the suspension then allowed to autolyse at  $37^\circ$ . When the cells were uniformly gram negative (4-5 days) the suspension was centrifuged. The supernatant which contained the nucleinase of the autolytic enzyme system and, in some cases, weak proteolytic activity, was decanted and the cells resuspended in water (80 ml) and 0.2 M phosphate buffer  $p_H$  8.0 (40 ml). Toluene (1 ml) was added and the suspension incubated at  $37^\circ$  until cellular disintegration occurred (4-7 days). The suspension was centrifuged at high speed and the turbid supernatant clarified by filtration through the SERRZ filter.

The hydrolytic activities of autolysates (4 ml) obtained in this manner against casein and peptone are shown in Table I.

TABLE I  
HYDROLYSIS OF CASEIN AND PEPTONE BY AUTOLYSATES OF *Staph. citreus* B 9

Time (hours)	<i>Staph. citreus</i> B 9 autolysate. Preparation:					
	R <sub>1</sub>		R <sub>2</sub>		R <sub>3</sub>	
	Increase in formol titration (ml 0.01 N NaOH)					
	Casein	Peptone	Casein	Peptone	Casein	Peptone
2.0			0.17	0.17	0.15	0.15
8.0			0.27	0.42		
16.5	0.41	1.52				
20	0.43	1.67			0.38	0.35
24			0.65	0.88		
41	0.89					
44					0.42	0.61
48			0.93	1.11		

b) *Staph. albus* (9238). Because of the rapidity with which autolysis of suspensions of this organism took place it was not possible to separate the enzymes of the autolytic system into the two distinct stages observed in the case of *Staph. citreus* B 9. Autolysis was allowed to proceed as follows:

Cells from 10 Roux bottles of a 48 hours culture of *Staph. albus* (9238) on peptone-glucose-agar were suspended in distilled water (120 ml) and 0.2 M phosphate buffer  $p_H$  7.5 (60 ml). The suspension was allowed to autolyse at  $37^\circ$  in the presence of toluene (2 ml). After 48 hours marked clearing had taken place and microscopic examination of stained smears showed that considerable disintegration of the cells had occurred. The suspension was centrifuged and the supernatant (solution F.A.) decanted. The deposit was suspended in distilled water (10 ml) and 0.2 M phosphate buffer  $p_H$  7.5 (20 ml) and incubated at  $37^\circ$ . After 10 days the suspension was centrifuged at high speed until the supernatant (S.A.) was completely clear.

The hydrolytic activities of such autolysates (4 ml) against the chemical substrates are shown in Table II.

The results recorded in Table II show that the main proteolytic activity was contained in solution F.A. These solutions were, therefore, employed in subsequent work. The fact that with the second solution (S.A.) the ratio hydrolysis of peptone:



hydrolysis of casein was, in general, greater than with solution F.A. suggested the presence in the autolysate of at least two proteolytic enzymes.

TABLE II  
HYDROLYSIS OF CASEIN AND WITTE PEPTONE BY  
AUTOLYSATES OF *Staph. albus* 9238

Preparation	Time (hours) at 37°	Peptone	Casein
		Increase in formol titration ml 0.01 N NaOH	
F.A.1	43.0	1.45	1.32
S.A.1	41.5	0.65	0.53
F.A.2	45.5	1.85	3.86
S.A.2	20.0	0.47	0.50
F.A.3	46.5	2.15	3.53
S.A.4	17.5	0.00	0.00
F.A.5	47.5	1.95	2.50
S.A.5	46.5	1.13	1.05
F.A.6	16.0	1.64	2.51
F.A.8	20.0	1.45	2.07

### *The Lytic Activity of Autolysates on Killed Cells*

The substrate of gram negative cells was prepared by re-heating a suspension of heat-killed staphylococci (B 9 or 9238), which had been rendered gram negative by the action of their own nucleinase at 80° for 30 minutes. After cooling, the suspension was centrifuged and the cells resuspended in a small volume of distilled water. 10 Drops of a uniform suspension of the substrate were added to each of a series of uniform tubes containing the crude enzyme solution (1 ml), 0.2 M buffer (pH range 4.0–9.5, 1.0 ml) and physiological saline (3.0 ml). After the addition of toluene (0.1 ml), the tubes were corked and incubated at 37°. After three days the corks were removed and the tubes replaced in the incubator for a further 24 hours in order to completely remove the toluene. The degree of lysis in each suspension was then determined as described above.

Such experiments showed that the autolysates were capable of bringing about the lysis of the gram negative cell bodies with pH optima which agreed closely with those obtained from the study of the spontaneous autolysis of *Staph. citreus* B 9 and *Staph. albus* 9238 respectively.

In contrast to the enzymes of the autolytic system responsible for the conversion of the gram positive cell to the gram negative state, the lytic fraction of the autolytic system was not species specific in nature. Thus, the lytic enzymes of *Staph. albus* 9238 or *Staph. citreus* B 9 was active against the gram negative forms of all gram positive organisms studied and readily lysed suspensions of the heat-killed, gram negative organism, *Bact. lactis aerogenes*.

### *Concentration of the Lytic Enzyme System*

Preliminary purification of the lytic system was obtained by fractional precipitation with ammonium sulphate.

a) *The lytic system of Staph. citreus* (B 9). The enzymes were completely precipitated at 0.75 saturation of ammonium sulphate. An equal volume of a saturated aqueous solution of ammonium sulphate was added to the autolysate at 5°. The small precipitate (precipitate a) which slowly separated was centrifuged off after several hours, dissolved in distilled water and the solution dialysed against distilled water at 5° until free from ammonium sulphate. The dialysed solution was then diluted with distilled water to

a volume equivalent to one tenth of the initial autolysate. The supernatant liquid was adjusted to 0.75 saturation of ammonium sulphate and the resulting precipitate (b) collected at the centrifuge after 4 hours at 0°, dissolved in distilled water and dialysed. In no case was any hydrolytic activity against either casein or peptone associated with this second precipitate. The activities of certain preparations of "precipitate a" (1 ml), determined against substrates of peptone and casein as previously described, are recorded in Table III.

TABLE III  
PROTEOLYTIC ACTIVITY OF THE LYtic ENZYME SYSTEM OF *Staph. Citreus* (B 9)  
CONCENTRATED BY AMMONIUM SULPHATE PRECIPITATION

Time (hours at 37°)	Enzyme Preparation									
	R1a				R2a				R3a	
	Peptone substrate		Casein substrate		Peptone substrate		Casein substrate		Peptone substrate	
	F	H	F	H	F	H	F	H	F	H
1.5	0.35	2.8								
2.5			0.18	0.7						
4.0	0.80	6.4			0.37	3.0	0.27	1.0		
4.5			0.28	1.1						
6.0	0.87	6.9	0.33	1.25			0.29	1.1		
21.5									0.91	7.3
23.0	1.70	13.9	0.85	3.15	0.84	6.7	0.76	2.9		
28.0									1.31	10.5
47.0	2.34	18.7	1.11	4.2	1.07	8.6	1.17	4.4	1.51	12.1

F = Increase in formol titration ml 0.01 N NaOH

H = % hydrolysis of substrate

These results reveal that the major part of the proteolytic activity (Table I) of the autolysates was concentrated in these precipitates and, therefore, that fractionation was accompanied by little loss in activity.

Such preparations were active in causing the lysis of killed, gram negative *Staph. citreus* B 9 with  $p_H$  optima 7.0–7.5 when examined according to the method previously described, but exhibited no activity against substrates of heat-killed gram positive cells.

b) *Staph. albus* 9238. Active autolysates were concentrated by precipitation with ammonium sulphate as in the case of the *Staph. citreus* autolysates. In contrast to *Staph. citreus*, the main bulk of the proteolytic activity was associated with the precipitate which separated at a concentration of 0.75 saturation of the salt (precipitate b). It is not, however, considered that this is due to any essential difference in the nature of the autolytic system of the two organisms, but to differences in such factors as the presence of inert material, concentration of the enzymes and duration of the initial autolysis.

After removing the ammonium sulphate by dialysis, the active precipitate was dissolved in a volume of distilled water equivalent to 1/6th the volume of the initial autolysate. The hydrolytic activities against peptone and casein of a series of such preparations (1 ml) are recorded in Table IV. Considerable variation was apparent in the ratio hydrolysis of casein: hydrolysis of peptone observed for the different enzyme preparations. Since the ratio of the rate of hydrolysis of one substrate to the rate of

References p. 399.

hydrolysis of a second substrate is constant for a given enzyme under similar conditions (IRVING, FRUTON, AND BERGMANN, 1941), such observations were taken as further indication that at least two proteolytic enzymes were associated with the staphylococcus lytic system.

TABLE IV  
PROTEOLYTIC ACTIVITY OF THE LYTIC ENZYME SYSTEM OF *Staph. albus* (9238) CONCENTRATED BY AMMONIUM SULPHATE PRECIPITATION

Enzyme preparation	Time (hours at 37°)	Peptone substrate		Casein substrate	
		Increase in formol titration ml 0.01 N NaOH	% Hydrolysis	Increase in formol titration ml 0.01 N NaOH	% Hydrolysis
A 3 b	18.0	1.85	37.1	2.58	24.4
A 5 b	22.0	2.22	44.5	2.85	27.0
A 6 b	22.5	2.28	45.7	3.65	34.5
A 7 b	23.5	0.83	20.9	0.90	8.5
A 8 b	19.5	1.35	27.0	0.63	15.6
A 9 b	19.5	2.90	58.1	3.45	32.8
A 10 b	17.5	2.55	51.1	2.60	24.7
A 11 b	19.0	1.80	36.1	2.00	19.0

As further experiments revealed that the purification of the active fractions was not achieved by refractionation with ammonium sulphate, alternative fractionation procedures were investigated.

#### *Concentration of Enzyme Activity by Ethanol Precipitation*

The proteolytic activity of the *Staph. citreus* autolysate (R 3) was concentrated by precipitation with ammonium sulphate. After dialysis, the active solution (R 3 a. 20 ml) was cooled to 0° and an equal volume of ethanol, cooled to -15° slowly added. During the addition of the ethanol, the temperature of the enzyme solution was brought to -15°. The flocculent precipitate (R. 3a. I) which separated was collected at the centrifuge after 30 minutes and dissolved in distilled water (5 ml). The second precipitate (R. 3a. II) which separated when ethanol (40 ml), cooled to -15°, was added to the supernatant at the same temperature was collected after 30 minutes and dissolved in distilled water (5 ml). It was found that these solutions (R. 3a. I and R. 3a. II) possessed approximately the same proteolytic activity against the chemical substrates. Thus, although on the basis of total nitrogen content (R. 3a. I., 0.15 mg N/ml; R.3a. II., 0.26 mg N/ml.) it may be considered that solution R. 3a. I contained the greater proportion of the enzyme, the distribution of the activity between the two precipitates together with the relatively small quantities of the enzymes involved, led to the conclusion that the limit where fractional precipitation may be used as a method of purification had been realized.

#### *Concentration of Enzyme Activity by Precipitation with Safranin*

ROBERTSON (1906) found that a precipitate was formed when solutions of safranin and trypsin were mixed together and he concluded that trypsin, acting as a weak acid, combined with the basic dye. It was shown by HOLZBERG (1913) that this precipitate possessed proteolytic activity of the same order as the original solution, but that a

considerable quantity of inert material was not precipitated by the safranin. It was not, however, found possible to purify trypsin by this method since all attempts to dissociate the trypsin-safranin complex resulted in the deactivation of the enzyme. Further studies by MARSTON (1923) showed that safranin or other azine bases precipitated 70% of the proteolytic activity of a pancreas extract leaving the remaining solution completely inactive. The enzyme-dye complex was dissociated, with considerable deactivation, by 0.2% hydrochloric acid. Extension of safranin precipitation to bacterial enzymes showed that the proteases of whole cultures of *Cl. histolyticum* and *Cl. sporogenes* were completely precipitated by the dye (BLANC AND POZERSKI, 1920) whereas the proteolytic enzymes of fungi were not precipitated (WAKSMANN, 1918).

When an equal volume of 0.5% aqueous safranin solution was added either to the crude staphylococcus autolysate or to an enzyme preparation concentrated by ammonium sulphate fractionation, the proteolytic activity was completely precipitated. The enzyme-dye precipitates possessed considerable hydrolytic activity against the chemical substrates, casein and peptone, whereas the residual supernatant solutions exhibited no action on these substances. In these experiments, the hydrolysis of the substrates was followed by measuring the increase in amino nitrogen by the copper method of POPE AND STEVENS (1939), for owing to the presence of the safranin, it was not possible to measure the proteolysis by means of the formol titration.

It was not however, possible to dissociate the enzyme dye precipitate over the  $p_H$  range 4–10. Below  $p_H$  4, complete loss of activity occurred.

### *Properties of the Proteolytic Enzymes of the Staphylococcus Autolytic System*

#### 1. $p_H$ optimum

The hydrolysis of Witte peptone and casein produced after 48 hours at different  $p_H$  values ( $p_H$  4.5–9.5) by a preparation (A3b) of the lytic system of *Staph. albus* 9238 was determined as follows:

A solution of the substrate (1 ml) and buffer (2 ml) with distilled water (4 ml) was approximately adjusted to the desired  $p_H$  (glass electrode) by the cautious addition of either 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. The enzyme solution (1 ml) was added, the solution adjusted to a volume of 10 ml with distilled water and the  $p_H$  determined. The amino nitrogen content of these solutions (1 ml) was determined by the formol titration immediately after their preparation and again after 48 hours at 37°.

The optimum  $p_H$  for the action of the proteolytic enzymes on both casein and peptone, as determined by this method was  $p_H$  7.5 in each case.

#### 2. Stability

The enzyme preparation (A5b, 5 ml), adjusted to the given  $p_H$  (glass electrode) and diluted to a volume of 10 ml, was immersed in a thermostat at 50°. At intervals of 30 minutes, aliquot fractions of the solution were withdrawn, adjusted to  $p_H$  7.0 and examined for hydrolytic activity against casein and Witte peptone, the extent of proteolysis being taken as the increase in the formol titration value after 48 hours at 37°.

The results showed that the lytic system was most stable towards denaturation by heat at  $p_H$  8.0. The concept previously put forward that at least two proteolytic enzymes are associated with the lytic fraction of the autolytic system, was substantiated by these results in that a parallel destruction of the casein and peptone activities was not observed. For example, when the enzyme solution was heated at acid  $p_H$  values the activity against peptone was decreased to a greater extent than that against casein.

In particular, when the enzyme was heated at 50° and p<sub>H</sub> 6.0 for 1 hour the peptone activity was almost completely removed, whereas the solution still exhibited 40% of its initial activity against casein. Furthermore, it was found that when preparations of the enzyme were allowed to stand at room temperature and p<sub>H</sub> 6.5 for several days the hydrolytic activity against peptone was almost completely destroyed though much of the casein activity was retained (Table V).

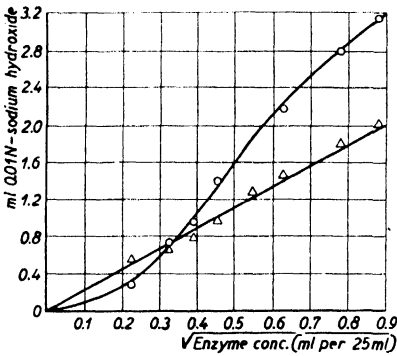


Fig. 6. Activity  $\sqrt{\text{concentration}}$  curves for the action of the proteases of the autolytic system of *Staph. albus* (9238).  $\circ$ — $\circ$  Activity against casein,  $\triangle$ — $\triangle$  Activity against Witte peptone.

water) was determined by measuring the increase in the formol titration value after incubation at 37° for 48 hours. Such experiments showed that the activity against peptone was proportional to  $\sqrt{\text{concentration}}$  (Fig. 6) as is the case with a single proteolytic enzyme, whereas the activity against casein was more complex, the hydrolysis of the substrate being greater at high concentrations than would be expected for a single enzyme. It is, therefore, concluded that the peptone-hydrolysing enzyme, itself unable to cause the hydrolysis of casein, brings about the hydrolysis of the split products resulting from the action of the second enzyme on this substrate.

4. The Effect of Enzyme Concentration on the Lysis of Heat-killed Gram Negative *Staph. albus* 9238

The lytic activity of increasing concentrations of the enzyme (preparation A6b) at p<sub>H</sub> 7.5 determined against a substrate of the gram negative forms of heat-killed *Staph. albus* 9238 revealed that the lysis was not directly proportional to the enzyme concentration (Fig. 7).

TABLE V

PROTEOLYTIC ACTIVITY OF *Staph. albus* LYTIC ENZYME (PREPARATION A3b) AFTER 5 DAYS AT ROOM TEMPERATURE

Time (hours at 37°)	Increase in formol titration ml 0.01 N NaOH	
	Peptone substrate	Casein substrate
23.0	0.00	0.75
31.0	0.00	0.94
48.0	0.19	1.30

3. The Effect of Enzyme Concentration on the Hydrolysis of Casein and Peptone

The hydrolysis of the casein and peptone substrates (1 ml) produced, at p<sub>H</sub> 7.5 (0.2 M phosphate buffer 0.5 ml), by increasing concentrations of the enzyme (preparation diluted to 1.0 ml with distilled

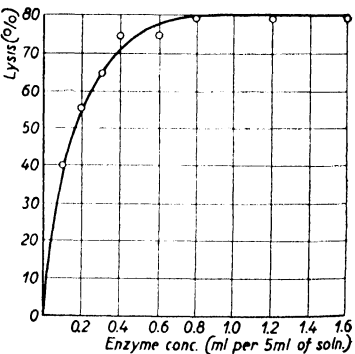


Fig.7. Activity-concentration curve for the action of the lytic enzyme of the autolytic system of *Staph. albus* (9238) on the gram negative cytoskeletons of heat-killed, *Staph. albus*.

5. The Effect of Oxidation and Reduction on the Proteolytic Enzymes

A solution of the lytic system of *Staph. albus* 9238 (Preparation A3b, 2 ml) was added

to (a) a saturated aqueous solution of hydrogen sulphide adjusted to  $p_H$  7.5 (2.0 ml) and (b) 3% hydrogen peroxide (2 ml). The solutions were allowed to stand at  $0^\circ$  for 24 hours and then examined for hydrolytic activity against peptone and casein. The results (Table VI) in conjunction with those of Table V showed that the lytic system of the staphylococcus autolysate was composed of at least two proteolytic enzymes. The fact that the enzyme solution in the presence of hydrogen sulphide was capable of hydrolysing casein, but exhibited no action against peptone, revealed that one of these enzymes was mainly responsible for the hydrolysis of protein fragments (such as peptones).

TABLE VI  
THE EFFECT OF OXIDISING AND REDUCING AGENTS ON THE ACTIVITIES OF THE PROTEOLYTIC ENZYMES  
OF *Staph. albus* (9238)

Time (hours at 37°)	Enzyme A 3a with hydrogen sulphide		Enzyme A 3a with hydrogen peroxide	
	Increase in formol titration ml 0.01 N NaOH			
	Peptone substrate	Casein substrate	Peptone substrate	Casein substrate
23.0	0.00	0.00	0.20	0.80
31.0	0.00	0.22	0.20	0.95
48.0	0.00	0.33	0.40	1.30

Correlation of the proteolytic activity of the enzyme preparation with the lytic activity against the gram negative forms of *Staph. albus* was provided by the fact that both activities were inhibited by reducing conditions. Identical results were obtained with further preparations of the lytic system of *Staph. albus* 9238 and of *Staph. citreus* (B 9).

6. The Effect of Concentration of Reducing Agent on the Action of the Enzyme System

a) On the lysis of gram negative forms of staphylococci. The effect of increasing concentrations of 0.1 M sodium thioglycollate (0.00 to 1.0 ml) on the activity of the enzyme preparation (A 12 b, 0.5 ml) (at  $p_H$  7.0) against the gram negative forms of *Staph. albus* (9238) was determined by the standard procedure. The results (Fig. 8) show that the inhibition of lysis increased with increasing concentration of sodium thioglycollate, but that complete inhibition was not realized.

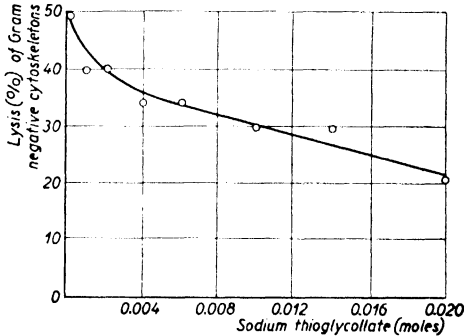


Fig. 8. Inhibition of the lytic enzyme of the autolytic system of *Staph. albus* (9238) with sodium thioglycollate

b) On the hydrolysis of Casein and Peptone

0.1 M sodium thioglycollate was added in increasing concentration to each of two series of tubes containing 1.5 ml of the enzymes preparation (A 12 b) and distilled water to 4.0 ml. After 24 hours at  $0^\circ$  5% Witte peptone (1 ml) was added to each tube of one series and 10% casein (1 ml) to each tube of the other. 1 ml quantities of each solution of the two series were withdrawn initially and after 48 hours at  $37^\circ$  and analysed for amino nitrogen by the formol titration method.

The results (Fig. 9) revealed that, whereas the proteolytic activity against both

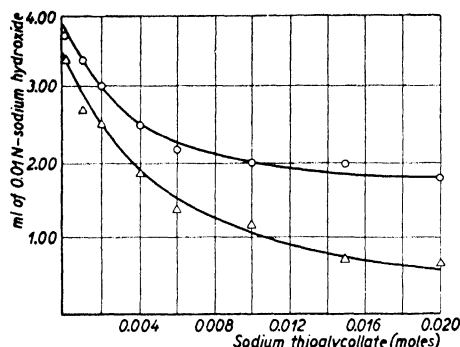


Fig. 9. Inhibition of the proteolytic enzymes of the autolytic system of *Staph. albus* (9238) with sodium thioglycollate.  $\circ$ — $\circ$  Casein substrate,  $\triangle$ — $\triangle$  Peptone substrate

system were adjusted to acid  $p_H$  values, turbidity resulted and a precipitate tended to separate.

The precipitate which separated when preparation (A 8 b, 8 ml) of the lytic system of *Staph. albus* (9238) was adjusted to  $p_H$  6.0 and allowed to stand for 24 hours, was collected at the high-speed centrifuge. The precipitate (A 8b, 6) was dissolved in distilled water (8 ml) and the supernatant adjusted to  $p_H$  5.0. After 2 hours at  $0^\circ$  the latter solution was centrifuged and the precipitate (A 8b, 5) dissolved in distilled water (8 ml). After adjusting the final supernatant (A 8b, P) to neutrality, the three solutions were examined for activity against the two substrates casein and peptone. The results (Table VII) show that although the ratio of the two proteolytic activities was different in each solution, complete separation, was not achieved and that the enzymes were only partially precipitated at the two  $p_H$  values.

TABLE VII  
FRACTIONATION OF THE PROTEOLYTIC ENZYMES OF *Staph. albus* (9238) BY ALTERATION OF  $p_H$

Enzyme	Increase in formal titration (ml 0.01 N NaOH) after 20 hours at $37^\circ$	
	Peptone substrate	Casein substrate
A 8b.6	0.45	0.13
A 8b.5	0.15	0.30
A 8b.P	0.80	1.05

*Acetone fractionation.* The enzyme solution (A 10 b, 35 ml) cooled to  $0^\circ$ , was brought to a concentration of 30% acetone by the addition of acetone (15 ml) cooled to  $-15^\circ$ . The resulting precipitate was collected (centrifuge) and dissolved in water (5 ml). In a similar manner, precipitates were collected at acetone concentrations of 50 and 80% and separately dissolved in distilled water (5 ml). These solutions (1 ml) were examined for hydrolytic activity against peptone and casein in the usual way. It was found (Table VIII) that the precipitates which separated at acetone concentration of 30 and 50% respectively were active in causing the hydrolysis of peptone but exhibited no activity against casein. Since it has previously been shown (Tables V and VI) that

Witte peptone and casein was considerably inhibited by sodium thioglycollate, the inhibition was not proportional to the concentration of the reducing agent and that complete inhibition was not obtained.

#### *Separation of the proteolytic activities*

*Adsorption.* Both enzymes were partially or completely adsorbed according to the concentration of the adsorbent, on  $C\gamma$  alumina at all  $p_H$  values and  $0^\circ$ , but the adsorbed enzymes could not be eluted by either acid or alkaline reagents.

*$p_H$  adjustment.* It was observed that when solutions of the staphylococcus lytic enzyme

an enzyme solution may be obtained which hydrolyses casein only, it is concluded that the staphylococcus lytic system contains two proteolytic enzymes one of which hydrolyses casein, but not peptone and the other peptone, but not casein. Although the precipitates which separated at acetone concentrations of 30% and 50% were without activity against the casein substrate (Table VIII) it was not possible to separate

TABLE VIII  
FRACTIONATION OF THE PROTEOLYTIC ACTIVITY OF THE  
STAPHYLOCOCCUS LYTIC ENZYME SYSTEM BY ACETONE PRECIPITATION

Enzyme activity precipitated at acetone concen- tration of (%)	Increase in formal titration (ml 0.01 N NaOH) after 20 hours at 37°	
	Peptone substrate	Casein substrate
30	0.57	0.00
50	0.52	0.00
80	1.20	1.75

the peptone hydrolysing enzyme by direct precipitation at the latter concentration for the addition of an equal volume of acetone to the concentrated lytic system resulted in the separation of a precipitate which was capable of hydrolysing both substrates. By further fractionation of this precipitate with acetone at concentrations of 50, 60, 70 and 80% it was possible to obtain the peptone hydrolysing enzyme free from activity against the casein substrate but in no case was the casein activity completely freed from activity against peptone. However, as the separated peptone hydrolysing enzyme was not capable of bringing about the lysis of a suspension of the gram negative forms of staphylococci whereas the enzyme solution containing the casein hydrolysing enzyme together with some peptone activity, caused almost complete lysis of a cell suspension at  $pH$  7.5 after 72 hours at 37°, it is concluded that the casein hydrolysing enzyme of the autolytic system is responsible for the initial lysis of the gram negative cell bodies. The protein fragments which result from the action of this enzyme on the cells are considered to be further hydrolysed by the action of the peptone hydrolysing enzyme.

#### DISCUSSION

The results of the foregoing experimental work reveal that the lytic fraction of the staphylococcus autolytic system contains two proteolytic enzymes, one of which is presumably adapted to the hydrolysis of the complex high molecular weight cell proteins, to smaller units. These units are then further broken down by the action of the second proteolytic enzyme which, alone, has no action on the intact gram negative cytoskeleton. These enzymes have no action on the corresponding strain of heat-killed, gram positive staphylococcus, and only play their part in spontaneous autolysis when cells have become gram negative by the action of the, as yet, undetermined species specific factor and the nucleinase. Since ribonucleinase-free proteolytic enzymes, such as trypsin, of animal origin are unable to lyse suspensions of heat-killed gram positive cells, but readily dissolve the gram negative forms of these organisms, it is concluded that the final disintegration of the gram negative cell bodies which occurs in autolysis, is a direct result of the action of the proteolytic enzymes.



The fact that the proteolytic enzymes of the staphylococcus lytic system are inhibited by hydrogen sulphide and sodium thioglycollate is of interest since most proteinases of the higher animals, plants and many bacteria are activated by compounds containing -SH groups. Bacterial proteolytic enzymes which are active under oxidising conditions, but which are inhibited by reducing agents, have, however, been described by MASCHMANN (1938) and WEIL, KOCHALATY, AND SMITH (1939). Furthermore, the results of GOEBEL AND AVERY (1929) revealed that the liberation of non-coagulable nitrogen from suspensions of autolysing pneumococci was much greater under aerobic than under anaerobic conditions and would indicate that the enzyme responsible was inhibited by the lack of oxygen.

It is fully realized that a complete picture of bacterial autolysis cannot be obtained from the evidence thus far obtained and it is intended to extend these investigations to other enzymes of the autolytic system.

One of us (M.S.) thanks the MEDICAL RESEARCH COUNCIL for a grant for personal assistance and expenses.

#### SUMMARY

The first major stage of the autolysis of certain strains of staphylococci involves the removal of ribonucleic acid from the cell by essentially the action of bacterial ribonuclease.

The second stage of the autolysis, involving the dissolution of the residual gram negative cytoskeletons, is brought about by a proteolytic enzyme system. Fractionation of the components of this system has revealed the presence of two proteolytic enzymes one of which hydrolyses casein but not peptone, and another which hydrolyses peptone but not casein. The latter enzyme is without action upon the gram negative staphylococcal cytoskeletons which are readily dissolved by the combined action of the two enzymes. It is suggested that the gram negative cytoskeletons are degraded by the casein-hydrolysing enzyme and that the resulting split products are further broken down by the action of the peptone-hydrolysing enzyme.

#### RÉSUMÉ

La première phase de l'autolyse de quelques souches des staphylocoques comprend l'élimination de l'acide ribonucléique de la cellule essentiellement sous l'action de la ribonucléinase bactérienne.

La seconde phase d'autolyse qui renferme la dissolution des cytosquelettes gram-négatifs résiduels se produit sous l'action d'un système d'enzymes protéolytiques. La séparation des composés du système a révélé la présence de deux enzymes protéolytiques, l'une hydrolysant la caséine et l'autre hydrolysant la peptone. Cette dernière enzyme est sans effet sur les cytosquelettes gram-négatifs des staphylocoques qui se dissolvent facilement sous l'action combinée des deux enzymes. On suggère ici que les cytosquelettes gram-négatifs sont dégradés par l'action de l'enzyme hydrolysant la caséine et, par suite, les produits de dégradation sont encore dégradés davantage par l'action de l'enzyme hydrolysant la peptone.

#### ZUSAMMENFASSUNG

Der erste Schritt in der Autolyse gewisser Stämme von Staphylokokken ist die Abspaltung von Ribonukleinsäure von der Zelle, hauptsächlich durch die Wirkung von bakterieller Ribonuklease.

Der zweite Schritt in der Autolyse, in dem die restlichen Gram negativen Cytoskelette in Lösung gehen, wird durch ein proteolytisches Enzym bewerkstelligt. Fraktionierung der Komponenten dieses Systems deutet auf die Gegenwart von zwei proteolytischen Enzymen. Das eine hydrolysiert Casein, aber nicht Pepton, das andere hydrolysiert Pepton, aber nicht Casein. Das letztere reagiert nicht mit Cytoskeletten der Gram negativen staphylokokken die bei der vereinten Wirkung beider Enzyme leicht löslich sind. Es wird vorgeschlagen, dass die Gram negativen Cytoskelete durch das Casein hydrolysierende Enzym abgebaut werden und die erhaltenen Abbauprodukte durch das Pepton hydrolysierende Enzym weiter abgebaut werden.

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## CHROMATOGRAPHIC INVESTIGATIONS OF AMINO ACIDS FROM MICRO-ORGANISMS

### I. THE AMINO ACIDS OF *CORYNEBACTERIUM DIPHTHERIAE*

by

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Study of the biochemistry of micro-organisms has revealed an essential similarity in general chemical composition and in enzymic processes between unicellular organisms and multicellular higher animals. The amino acid content of bacterial cells and their protein has, however, been relatively little studied owing to technical limitations. These limitations have now been largely overcome by the introduction of paper partition chromatography by CONSDEN, GORDON, AND MARTIN (1944), a technique which enables all the amino acids in a few milligrams of any mixture to be characterized and roughly estimated.

The objects of the present study were (a) to characterize the amino acids, both free (soluble) and bound (insoluble), in *C. diphtheriae*, (b) to determine the gross effect of certain variations in the culture medium on the bound amino acid content of the cells and (c) to investigate changes in the amino acids of synthetic media caused by growth therein of *C. diphtheriae*.

Relatively few publications are available recording complete amino acid analyses of bacteria. Early figures for the arginine, histidine, lysine and cystine contents of various bacterial species have been summarized by PORTER (1946) and by CAMIEN, SALLE, AND DUNN (1945). Only two investigations on the amino acids of *C. diphtheriae* have been reported, in both cases only arginine, histidine, lysine and cystine having been determined. TAMURA (1914) examined a protein fraction from the organisms, and HIRSCH (1931) used whole cells.

More complete amino acid analyses, by microbiological assay, of hydrolysates of whole cells of various microorganisms and reports on effects of change in composition of the medium on the levels of individual amino acids were published by CAMIEN, SALLE, AND DUNN (1945) and by STOKES AND GUNNES (1946). POLSON (1948) investigated by paper partition chromatography the amino acids in hydrolysates of *Escherichia coli*. In all of these studies, results are given for whole cells and no distinction is made between free and bound amino acids. FREELAND AND GALE (1947), GALE (1947), and TAYLOR (1947) gave figures for both the free and the combined arginine, lysine, histidine, tyrosine and glutamic acid of various microorganisms, determined by specific amino acid decarboxylases, and examined the combined amino acids of *E. coli*. and *Aerobacter aerogenes*

grown on a variety of media. The free amino acids were determined after disruption of the cells in boiling water — subtraction of this figure from the total amino acid in the acid hydrolysate gave the bound amino acid.

In the present study, free amino acids and alcohol-soluble peptides were extracted from washed wet *C. diphtheriae* cells by ethanol. The extracts were examined by two dimensional paper partition chromatography, as were also the amino acids present in hydrolysates of the alcohol-insoluble residues.

Chromatograms were also made of two media of known amino acid composition before and after growth of *C. diphtheriae*. Since the work was done, a similar investigation with single-dimensional chromatograms was reported by LINGGOOD AND WOIWOD (1948), who followed changes in peptone broth and casein hydrolysate after growth of *C. diphtheriae*.

#### METHODS

*C. diphtheriae*, P.W. 8 (substrain "Toronto") was used throughout this work. It was cultivated at 34° C for 7 days, either on synthetic media of known composition (Table I) or on casein hydrolysates of known iron content containing 0.1 g of casein nitrogen/100 ml supplemented by 0.15 g glycine/100 ml (HOLT, 1948).

TABLE I  
COMPOSITION OF SYNTHETIC MEDIA FOR CULTIVATION OF *C. diphtheriae*

	g/100 ml of medium	
	Medium 1	Medium 2
Tryptophan . . . . .	0.01	0.01
Glycine . . . . .	0.05	0.1
Valine . . . . .	0.05	0.05
Alanine . . . . .	0	0.05
Leucine . . . . .	0.05	0.1
Glutamic acid . . . . .	0.1	0.25
Methionine . . . . .	0.04	0.04
Tyrosine . . . . .	0.04	0.04
Arginine . . . . .	0.03	0.03
Histidine . . . . .	0.02	0.02
Lysine . . . . .	0.02	0.02
Aspartic acid . . . . .	0.05	0.05
Proline . . . . .	0.06	0.06
Phenylalanine . . . . .	0.04	0.04
Cystine . . . . .	0.01	0.01
$\beta$ -Alanine . . . . .	0.0005	0.0005
Pimelic acid . . . . .	0.0005	0.0005
Nicotinic acid . . . . .	0.001	0.001
NaCl . . . . .	0.5	0.5
$\text{KH}_2\text{PO}_4$ . . . . .	0.2	0.2
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	0.03	0.03
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	0.0004	0.0004
Maltose . . . . .	0.9	0.9

After harvesting, the cells were washed three times by centrifugation from saline containing 0.85 g. NaCl/100 ml, were extracted 3 or 4 times by standing overnight at room temperature under ethanol, and were then extracted 3 times with acetone. The ethanol extracts were pooled and concentrated *in vacuo* to a small bulk; the suspended fat was removed by ether extraction, and the remaining aqueous solution filtered when necessary.

The cell residues remaining after ethanol and acetone extraction were dried and hydrolysed at 100° C under reflux for 24 h with 6 N-HCl; the HCl was removed *in vacuo*, and the solids were dis-

solved in sufficient water to give a total N content of about 1 mg/ml. Nitrogen was estimated by the standard micro-kjeldahl procedure, one sample of the cells being taken for dry weight determination at the same time.

### CHROMATOGRAPHIC TECHNIQUES

With a few exceptions, the techniques and apparatus were the same as those described by DENT (1948). The troughs for holding solvents were either of glass or of polythene tubing. The filter paper used at the beginning of the work was Whatman No. 1, but this was later changed to No. 4, as the solvents travelled faster over No. 4 grade and better separation was obtained in the "collidine" run.

Water-saturated phenol was used as the first solvent, followed by a mixture referred to in this text as "collidine". This mixture was prepared as suggested by Dr. C. E. DENT (unpublished), from equal parts of *sym*-collidine and a commercial mixture of the 2 : 4 and 2 : 5 lutidine isomers. Great care was taken to ensure that the "collidine" mixture was saturated with water when used, a precaution necessitated by the marked variation in composition of the saturated solution caused by small temperature changes in the region of 18°C; an unsaturated solvent produced poor separation of the amino acids. The ninhydrin colour was developed by heating for 5 minutes at 110°C.

The individual amino acids were generally identifiable by their position in relation to other known amino acids; "the pattern of the spots" (DENT, 1948) being the most satisfactory method of characterization. Where any doubt existed, further tests, advocated by DENT (1948), were performed to establish the identity of the amino acid in question. Although already published by DENT, these tests are considered to be of such importance that they will be listed again.

- (1) Matching against the suspected amino acid run under identical conditions.
- (2) Specific colour reactions — *e.g.*, PAULY, SAKAGUCHI.
- (3) Change of reaction of atmosphere surrounding filter paper during solvent runs; particularly useful in testing basic substances whose speed of running in phenol is markedly slowed on changing from a basic to an acidic atmosphere.
- (4) Chemical treatment. This may involve acid or alkaline hydrolysis, oxidation with peroxide, or any other suitable treatment before repeating a chromatographic separation of the amino acid or its reaction products.
- (5) Change of solvent. This made possible, for example, the separation of the leucine isomers

(see Results) which is impossible with the conventional phenol-collidine two-dimensional chromatograms (DENT, 1948).

- (6) Isolation. In the case of a completely unknown substance, final characterization will only be achieved after isolation and purification. Provided sufficient quantities of material are available, the substance can be purified by any suitable chemical procedure. Isolation by cutting out and eluting spots or bands from two or one-dimensional chromatograms can be carried out on small quantities of material (DENT, 1947, b AND CONSDEN, GORDON, AND MARTIN, 1947).

In the present study elution was carried out as suggested by CONSDEN, GORDON, AND MARTIN (1947). Sufficient eluate for preparative purposes could be collected by the following arrangement (see Fig. 1). Triangular glass plates A, placed with their right angles uppermost, were stuck to a wooden burette clamp B, so that on closing the clamp the two surfaces made contact. The filter paper strips C, were hung from a sheet of wet filter paper, D, immersed in a trough, E, containing the eluting liquid; their bottom tips being between the two plates, A. The eluting liquid, dripping from the strips, collected between the plates, ran down the inclined edge and finally dripped off the acute angle into a container F. When the elution was completed, the plates were separated by unscrewing the clamp, and the liquid remaining between the plates all ran into the container. It was found that elution proceeded faster if the whole apparatus was placed in a jar saturated with the eluting solvent, but this was not essential.

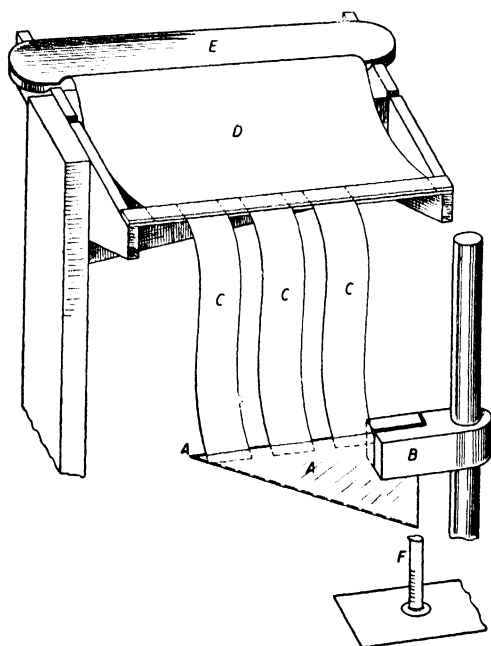


Fig. 1. Diagram of apparatus for washing material off "cuts" from chromatograms on a preparative scale

## ESTIMATION OF AMINO ACIDS

Rough estimations of the amount of amino acids present in a mixture were made by comparing the colour intensity (matched against an arbitrary scale) and size of the spots with those given by known amounts of pure amino acids run under identical conditions. These criteria as employed gave consistent results, with an accuracy of  $\pm 50\%$ , provided amounts of amino acids were chosen which gave sub-maximal colour intensities. Above a certain amount of amino acid, a maximal colour is reached, and comparison of size of spot alone does not give a satisfactory estimation in two dimensional chromatograms. FISHER, PARSONS, AND MORRISON (1948) claim that with one-dimensional strips the area of the spot is proportional to the logarithm of the amino acid content, but this was not found to be the case with two dimensional chromatograms.

## RESULTS

## SEPARATION OF LEUCINE ISOMERS

Before investigating the amino acids present in *C. diphtheriae*, it was considered essential to establish a satisfactory method of separating the leucine isomers. EDMAN (1945) and WRETLINE (1947) employed a pyridin-amyI alcohol-water mixture instead of collidine, but obtained poor separation of the isomers. Benzyl alcohol-water and n-butanol-water solvents have been used by CONSDEN, GORDON, AND MARTIN (1944) and by CONSDEN, GORDON, MARTIN, AND ROSENHEIM (1945) in one dimensional chromatograms, but in this laboratory the separation produced by these solvents was not considered satisfactory.

Amyl alcohol-water gave better separation, although all the Rf values were lower than those given by CONSDEN, GORDON, AND MARTIN (1944). The actual distance run by the amino acid down the paper was increased by allowing the solvent to run for several days and to drip from the bottom of the paper. By this means commercial amyl alcohol produced satisfactory separation of leucine, isoleucine and phenylalanine (Table II). However, since the results could not be repeated with different samples of solvent, and since commercial amyl alcohol consists of an unspecified mixture of the different isomers, it was decided to investigate individual isomers. Table II shows the results with 4 isomers, either in the absence or the presence of diethylamine vapour. The presence of this substance was found to increase the distance run by phenylalanine

TABLE II

SEPARATION OF AMINO ACIDS IN AMYL ALCOHOL-WATER ON ONE-DIMENSIONAL FILTER PAPER CHROMATOGRAMS. SOLVENT ALLOWED TO RUN OVER END OF PAPER. (NUMBERS INDICATE DISTANCE TRAVELLED RELATIVE TO MENISCUS)

Amyl alcohol used	Diethylamine vapour	Position of							Shape of spots
		leucine	iso-leucine	nor-leucine	phenylalanine	valine	methionine	tyrosine	
Commercial	—	1	0.81		0.46		0.64		Variable, sometimes v. elongated
Normal	—	1	0.86		0.77	0.42	0.52	0.37	Oval
Normal	+	1	0.85		1	0.52	0.52	0.29	Oval
Iso-	—	1	0.82		0.8	0.36	0.53	0.44	Elongated
Iso-	+	1	0.8		0.93	0.39	0.39	0.24	Elongated
Active	—	1	0.84		0.84		0.43		Oval
Tertiary	—	1	0.85		1	0.48	0.43	0.48	Round
Tertiary	+	1	0.86	1.2	1.11	0.51	0.65	0.45	Round

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and methionine, allowing them also to be separated. The correct amount of diethylamine must be determined for each individual apparatus, as an excess causes a general purple background to appear on development of the ninhydrin colour. Considerable variation in the shape of the spots was found with different amyl alcohols, and the final choice, tertiary amyl alcohol, was selected because it produced the least elongated spots and the best separation. By this means, the leucine isomers, phenylalanine, methionine and valine could be separated and identified in a complex amino acid mixture (e.g., protein hydrolysate), since all the other naturally-occurring acids ran considerably more slowly along the paper (Fig. 2). The  $R_f$  values for tyrosine, the amino acid with the next highest speed of running are included in Table II. Fig. 3 shows results obtained from hydrolysates of insoluble cell residues of *C. diphtheriae*.

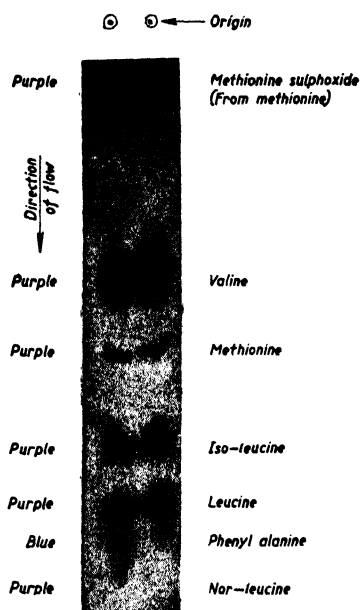


Fig. 2. Amino acid mixtures run on one-dimensional chromatogram for 3 days; solvent, tertiary amyl alcohol-water + diethylamine vapour

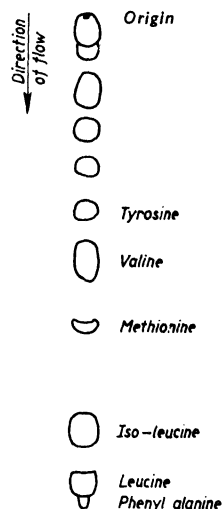


Fig. 3. Hydrolysed ethanol-insoluble residues of *C. diphtheriae* (32  $\mu$ g N) run on one-dimensional chromatogram for 3 days; solvent, tertiary amyl alcohol-water + diethylamine vapour

#### INSOLUBLE CELL RESIDUES OF *C. diphtheriae*

Cells of *C. diphtheriae* were grown in 3 different media, which are described in the Method section. The amino acids identified in the acid hydrolysates of the residues remaining after alcohol and acetone extraction of the specimens examined, are listed in Table III, in which a rough quantitative comparison is included. Fig. 4 shows diagrammatically a typical square obtained from an amount of hydrolysate equivalent to 48  $\mu$ g N.

Although the method of estimation is far from accurate, it shows the constancy in amino acid composition of the cells grown on various media. This constancy was even

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TABLE III

AMINO ACIDS FOUND IN HYDROLYSATES FROM INSOLUBLE CELL RESIDUES OF *C. diphtheriae*  
(Figures show approximate concentration of amino acids, expressed as amino acid N  $\times$  100/total N.  
- indicates that no estimation has been made.)

Amino acid	<i>C. diphtheriae</i> grown on		
	Casein hydrolysate 0.14 $\mu$ g Fe/ml	Casein hydrolysate 1.4 $\mu$ g Fe/ml	Synthetic medium 2 1.4 $\mu$ g Fe/ml
Aspartic acid . . . . .	4	4	5
Glutamic acid . . . . .	9	8	8
Serine . . . . .	2	2	3
Glycine . . . . .	5	5	5
Threonine . . . . .	1	1	1
Alanine . . . . .	8	8	8
Valine . . . . .	7	7	7
Leucine . . . . .	4	4	4
Isoleucine . . . . .	4	5	4
Phenylalanine . . . . .	3	3	3
Cystine . . . . .	-	-	-
Methionine . . . . .	3	-	-
Tyrosine . . . . .	2	2	2
Histidine . . . . .	4	-	-
Proline . . . . .	5	5	5
Hydroxyproline . . . . .	1	1	1
Arginine . . . . .	10	10	10
Lysine . . . . .	5	-	5
Total N content, percent dry weight . . . .	12.9	13.6	7.1

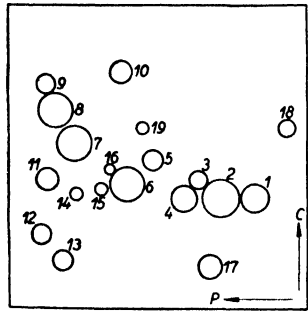


Fig. 4. Acid hydrolysate of insoluble residues of *C. diphtheriae* (48  $\mu$ g N) run on two dimensional chromatogram. Solvents, phenol  $\text{NH}_3$  + collidine. 1. aspartic acid, 2. glutamic acid, 3. serine, 4. glycine, 5. threonine, 6. alanine, 7. valine, 8. leucine and methionine, 9. phenylalanine, 10. tyrosine, 11. proline, 12. arginine, 13. lysine, 14. methionine sulphoxide, 15. histidine, 16. hydroxyproline, 17. unidentified, 18. cysteic acid, 19. unidentified

better appreciated in the comparison of individual squares, which showed remarkable similarity in colour-depth and size of amino acid spots from hydrolysates of different cell residues, taking total nitrogen content as the basis for comparison (12, 18, 32, and 120  $\mu$ g N).

Besides the known amino acids, an unknown purple spot (17) was consistently found in all fresh hydrolysates in a position corresponding very closely to that of ethanolamine phosphoric acid in the "map of spots" published by DENT (1948). This spot from *C. diphtheriae* has not yet been identified; it is unchanged in position after treatment with alkaline phosphatase in contrast to ethanolamine phosphoric acid; it is also unaffected by hydrogen peroxide and is thus not one of the sulphur-containing amino acids such as cystine, lanthionine or cystathione which are located in about the same position on the square.

A brown spot (19) turning purple on standing was not identified as any known substance; its speed of running was unchanged by using an acid atmosphere, so that it could not have been a basic substance such as glucosamine (DENT, 1948). It did not appear consistently and has not been further investigated.





The supernatant liquid obtained from a large batch of toluene-treated *C. diphtheriae* cells which had stood at room temperature for several months presented similar squares. A yellow-brown spot (24) was also found in squares from this liquid or from its hydrolysate, but has not been further investigated.

#### CHANGES IN SYNTHETIC MEDIA FOLLOWING GROWTH OF *C. diphtheriae*

Attempts to find a suitable synthetic medium for growth of *C. diphtheriae* had produced a medium (medium I, see Methods) which maintained only poor growth. Changes

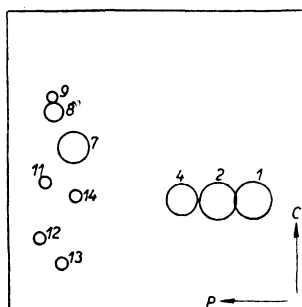


Fig. 6A

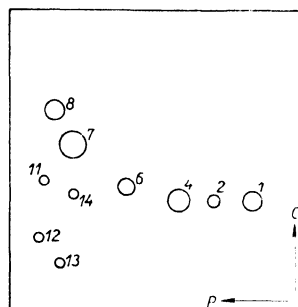


Fig. 6B

Fig. 6. Synthetic medium I (15  $\mu$ l). A before growth, B after growth, of *C. diphtheriae*. Run on two-dimensional chromatogram. Solvents, phenol- $\text{NH}_3$ , collidine. 1 aspartic acid, 2 glutamic acid, 4 glycine, 6 alanine, 7 valine, 8 leucine and methionine, 9 phenylalanine, 11 proline, 12 arginine, 13 lysine, 14 methionine sulfoxide

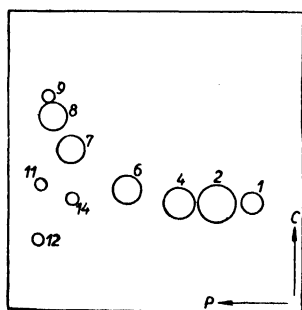


Fig. 7A

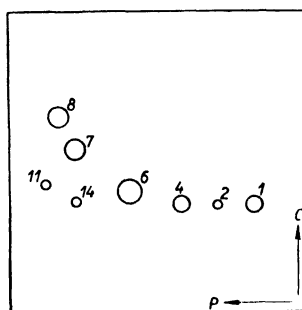


Fig. 7B

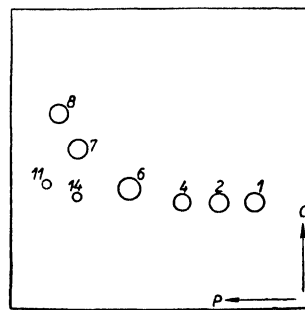


Fig. 7C

Fig. 7. Synthetic medium II (10  $\mu$ l). A before growth, B after growth of *C. diphtheriae*, C after growth hydrolysed 6 N-HCl. Solvents and numbers as in Fig. 6

in amino acid content caused by growth of the organism were followed by comparing chromatograms made from equal volumes (15  $\mu$ l) of the medium before and after growth. There was, as expected, a general fall in concentration of all amino acids, but aspartic acid and particularly glutamic acid were preferentially removed during growth of the organism (see Fig. 6). Alanine appeared in the culture filtrate, although not originally present in the medium.

Following this result, the basal medium was changed by alteration of the proportion of various amino acids, notably by addition of alanine and of more glutamic acid

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(medium 2). Better growth of the organism was maintained, and the chromatograms obtained from 10  $\mu$ l (Fig. 7 A and B) show that the drop in glutamic acid was even more striking than in the case of medium 1. Aspartic acid was relatively unchanged, but glycine showed a considerable fall in strength, while alanine in this case showed a slight fall. Acid hydrolysis of a sample of the culture filtrate (Fig. 7c) produced a slight rise in the strength of the glutamic acid spot, but the spot remained far from its original size and intensity, showing that all the glutamic acid lost during growth could not be accounted for as glutamine or any other hydrolysable derivatives still present in the medium. Application of larger amounts (90  $\mu$ l) of culture filtrate to squares did not show the presence of any ninhydrin-reacting substances other than those composing the medium.

#### DISCUSSION

Changes in media following the growth of *C. diphtheriae* have been investigated by LINGGOOD AND WOIWOD (1948). Using one-dimensional chromatograms, they obtained results with casein hydrolysate essentially similar to those reported in this paper with synthetic medium 1 — namely, preferential utilization of aspartic acid and glutamic acid and appearance of alanine. The glutamic acid content of this synthetic medium was of the same order as that of the casein hydrolysate used by LINGGOOD AND WOIWOD, but alanine was not present. Raising the glutamic acid level and adding alanine in amount approximately double that present in the casein hydrolysate, resulted in much improved bacterial growth. Some alanine was used up, aspartic acid showed a normal drop in concentration but even more glutamic acid was used than previously. At present it is not possible to say which variation in composition of the modified medium was responsible for the improved growth, nor is it possible to account for the assimilation of such comparatively large amounts of glutamic acid. In medium 1, glutamic acid might have been converted to alanine by transamination, but as sufficient alanine appeared to be present in the modified medium, this explanation is unlikely. The glutamic acid content of the cell residues (8% of total N as glutamic acid N) is certainly higher than that reported for other bacteria by FREELAND AND GALE (1947) whose average value for combined glutamic acid N of 9 types of bacteria was 5.3% of total N. The amount of free glutamic acid in the internal environment (as shown by chromatographic analysis of the ethanol extracts) was not unduly high, and showed no indication of a process of concentration such as was reported by TAYLOR (1947) for Gram-positive organisms. GALE AND MITCHELL (1947), during their studies on glutamic acid assimilation of *Strep. faecalis*, found a disappearance of glutamic acid from the internal environment of the organism which could not be accounted for by protein or peptide synthesis. Possibly glutamic acid is involved in some metabolic processes as yet unknown.

The rough composition put forward for the insoluble cell residues presents nothing of particular interest, except for the relatively high glutamic acid content. Possibly the arginine content (10% of total N as arginine N) should also be commented on in the light of the findings of FREELAND AND GALE (1947), who suggest that Gram-positive organisms have bound arginine levels of approximately half this figure; the average value for Gram-negative organisms is, on the other hand, the same as that found for *C. diphtheriae*. This organism does not exhibit many of the characteristic properties of Gram-positive organisms, and has in fact been classified by DUBOS (1945) as Gram-variable.

Comparison of these figures with those previously published for *C. diphtheriae* is difficult. The values given by HIRSCH (1931) were determined on whole cells and show no agreement with the present findings (Table IV). This is not surprising; the figures of FREELAND AND GALE (1947) for 6 micro-organisms show very variable agreement between the total amino acid contents estimated on hydrolysates of whole cells and the figures corrected for free amino acids in the cells. The analyses given by TAMURA (1914) certainly do not refer to whole cells; they were made on the insoluble residue remaining after extraction with alcohol and acid.

The method of estimation used is admittedly rough, and the results are only presented as the nearest whole number; the constancy in the amino acid composition obtained in cells grown on various media is however worthy of note. The media were

TABLE IV  
COMPARISON OF LEVELS OF ARGININE, LYSINE AND HISTIDINE FOUND IN *C. diphtheriae* BY PARTITION CHROMATOGRAPHY WITH THOSE GIVEN BY HIRSCH (1931) AND TAMURA (1914)

	% of total N		
	By chromatography	HIRSCH	TAMURA
Arginine N . . . . .	10	15.9	10.6
Lysine N . . . . .	5	9.7	4.9
Histidine N . . . . .	4	6.0	1.0

chosen according to their ability to cause toxin formation by the organism. The casein hydrolysate containing 1.4  $\mu$ g of iron/ml, produces maximum growth of the organism, with no toxin formation. Reduction of the iron content by 1/10th causes the organism to excrete large amounts of toxin into the medium — at the same time coproporphyrin is also excreted, and there occurs a fall in the intracellular haem pigments, notably of cytochrome b (PAPPENHEIMER AND HENDEE, 1947; RAWLINSON AND HALE, 1949). PAPPENHEIMER AND HENDEE suggested that the toxin is a portion of the cytochrome molecule and, in the absence of sufficient iron for cytochrome formation, both toxin and porphyrin are excreted. Preliminary investigations on a sample of partially purified toxin (kindly provided by Dr. G. R. TRISTRAM) show that its amino acid composition is quite different from that of the insoluble cell residues. The loss by the cell of large quantities of a highly specific protein in the form of toxin might be expected to be reflected in a considerable change in amino-acid composition of the residual protein, but such was not the case. Changing the source of amino acids from a casein hydrolysate to a synthetic mixture of amino acids, present in different proportions from those of the casein hydrolysate, also made no difference to the amino acid composition of the cell residues. These results conform with those of other authors, who find no difference in the amino acid composition of bacterial cells grown on different media (CAMIEN, SALLE, AND DUNN, 1945; STOKES AND GUNNESS, 1946; FREELAND AND GALE, 1947).

The ethanol extracts of *C. diphtheriae* contain 6 ninhydrin-reacting substances not present in the cell proteins. Four of these are known amino acids, of which two,  $\alpha$ - and  $\gamma$ -amino butyric acids, were found by DENT (1947 a and b; 1948) in plant extracts and in fluids such as blood and urine, but they have not been found in any protein. A spot corresponding to  $\alpha$ -amino butyric acid was found by POLSON (1948) in hydroly-

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sates of whole *E. coli*; ACKERMANN AND KIRBY (1948) have not agreed with its identity and found that it behaved like pantonine.

No evidence has been obtained as to the identity of the other ninhydrin-reacting substances. The basic material mainly responsible for spot (23) is not identical with the basic substance "fast arginine" found by DENT (1948) in pathological blood and urines, as it is slowed to a greater extent in phenol/acetic acid than is DENT's material. Many basic substances, including cadaverine, give spots in approximately this position when run in phenol-ammonia. In the original unhydrolysed alcoholic extracts there was evidently a mixture of basic substances, one of which was similar to "fast arginine", but this disappeared on hydrolysis.

The presence of unidentified ninhydrin-reacting substances in both the ethanol extracts and insoluble cell residues of *C. diphtheriae* is not surprising. The discovery of hitherto unknown amino acids in various antibiotics has shown that microorganisms can produce a variety of amino acids combined in various forms and not usually known to be present in proteins.

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#### SUMMARY

The approximate amino-acid composition of hydrolysates of ethanol-extracted *C. diphtheriae* cells was determined by paper partition chromatography.

Change of culture medium had no effect on the composition of the cell residues.

A new single dimensional technique for separation of the leucine isomers is described.

Two unidentified ninhydrin reacting substances were found in hydrolysates from the insoluble cell residues.

Ethanol extracts from the organisms contained most of the known amino acids found in the insoluble residues, but in addition contained  $\alpha$ -amino butyric acid,  $\gamma$ -amino butyric acid, hydroxylysine,  $\beta$ -alanine and two unidentified acid-stable ninhydrin-reacting substances.

#### RÉSUMÉ

La composition approximative en acides aminés d'hydrolysats de cellules de *C. diphtheriae* extraites à l'alcool a été déterminée par chromatographie de partage sur papier. Le changement de la nature du milieu de culture n'a pas d'influence sur la composition des résidus cellulaires. Une nouvelle technique de séparation unidimensionnelle des isomères de la leucine est décrite. Deux substances réagissant avec la ninhydrine et non encore identifiées ont été trouvées dans les hydrolysats des cellules extraites à l'éthanol.

Les extraits éthanoliques contiennent la plupart des acides aminés connus que l'on rencontre dans la partie insoluble, mais renferment en outre l'acide  $\alpha$ -aminobutyrique, l'acide  $\gamma$ -aminobutyrique, l'hydroxylysine,  $\beta$ -alanine et deux substances stables vis-à-vis des acides réagissant à la ninhydrine et encore non identifiées.

#### ZUSAMMENFASSUNG

Zellen von *C. diphtheriae* wurden mit Äthanol extrahiert, hydrolysiert und die Zusammensetzung des Hydrolysates annähernd durch "Partition Chromatography" auf Papier bestimmt.

Eine Veränderung der Nährlösung hatte keinen Einfluss auf die Zusammensetzung der Zellreste.

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Eine neue eindimensionale Arbeitsweise zur Trennung der Isomeren des Leucins wurde beschrieben.

In Hydrolysaten von unlöslichen Zellresten wurden zwei Substanzen gefunden, die mit Ninhydrin reagieren, aber bis jetzt nicht identifiziert wurden.

Die Äthanolauszüge der Organismen enthielten die meisten der bekannten Aminosäuren, die auch in den unlöslichen Rückständen gefunden worden waren, ausserdem aber noch  $\alpha$ -Aminobuttersäure,  $\gamma$ -Aminobuttersäure, Hydroxylysin,  $\beta$ -Alanin und zwei säurebeständige, mit Ninhydrin reagierende Substanzen, die nicht identifiziert wurden.

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## ACTION DES ULTRASONS SUR UNE OXYDASE

par

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Les ultrasons (U.S.) provoquent la désintégration des cellules, et, tout particulièrement, des microorganismes. Cette propriété peut être utilisée et l'a été pour l'obtention d'extraits microbiens riches en antigènes ou en enzymes. Mais cette désintégration, due aux effets mécaniques de la cavitation, est accompagnée d'une activité chimique des U.S.

Dans des publications antérieures, deux d'entre nous ont mis en évidence les modifications importantes que subissent les protéines, ainsi que des composés plus simples<sup>1, 2</sup>, et ont proposé une explication du mécanisme de l'action oxydante des U.S.<sup>3, 4, 5</sup>. Ce mécanisme serait le suivant: les charges électriques qui prennent naissance sur les parois des bulles de gaz engendrées par la cavitation, provoquent des effluves (accompagnées de luminescence). Il s'ensuit une forte ionisation de l'eau et la formation de radicaux libres (comme dans le cas des rayons X) de grande activité chimique. Ainsi les noyaux aromatiques sont attaqués, les composés éthyléniques sont saturés, diverses oxydations ont lieu, etc. D'une manière générale, l'activité mécanique (p. ex., destruction des microbes) des U.S. est plus rapide que ces effets chimiques.

On pouvait s'attendre à ce que des enzymes soient inactivés par les U.S. C'est ce que nous avons essayé de contrôler en nous adressant à un enzyme assez labile, une polyphénoloxydase du champignon *Agaricus campestris*.

Nos recherches nous ayant montré, d'autre part, que les actions chimiques des ultrasons sont supprimées en absence de cavitation et aussi presque toujours lorsqu'on opère en présence d'hydrogène dans la phase gazeuse, nous avons également essayé de nous rendre compte si, dans ce cas, on observe cet effet protecteur.

Nous croyons que ces expériences présentent un certain intérêt parce qu'elles montrent que l'on doit tenir compte d'un éventuel effet destructeur des ultrasons, lorsqu'on s'adresse à eux pour obtenir un extrait contenant des enzymes.

## TECHNIQUES

1. *Préparation des solutions enzymatiques.* Nous avons suivi ici la méthode de KEILIN ET MANN<sup>6</sup>, en partant de 5 kg de champignons frais (*Agaricus campestris*). Nous avons été amenés toutefois à employer des quantités d'acétate de plomb et de gel de phosphate tricalcique légèrement différentes de celles utilisées par ces auteurs, et nous avons arrêté nos essais de purification avant la précipitation par l'acétone car, à ce stade, nous observions des altérations de l'enzyme. Nous avons jugé que le matériel obtenu, qui possédait

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une activité enzymatique 200 fois supérieure à celle du jus initial, était suffisamment purifié pour pouvoir servir dans les expériences envisagées.

2. *Mesure de l'activité oxydasique.* Nous avons utilisé une méthode colorimétrique, basée sur l'oxydation du pyrogallol en purpurogalline, similaire à la méthode introduite par WILLSTÄTTER<sup>7</sup>.

On ajoute à une solution de 1 g de pyrogallol bisublimé dans 250 ml d'eau distillée (exempte de cuivre) une quantité de solution enzymatique calculée de manière à obtenir entre 2 et 5 mg de purpurogalline. On agite à l'air dans une ampoule à décantation durant 5 min à 20°; l'oxydation est alors arrêtée par 5 ml d'une sol. d'acide sulfurique à 20%. On extrait alors par de l'éther chimiquement pur la purpurogalline formée. On complète à 100 ml avec ce solvant et on prélève la quantité nécessaire pour la mesure colorimétrique en ayant soin de filtrer le liquide sur un petit entonnoir à longue tige garni d'un filtre sans cendres (DURIEUX), afin de retenir l'eau contenue dans l'éther et de clarifier éventuellement la solution. Les mesures colorimétriques ont été faites avec un électrophotomètre à cellules au Sélénium montées en opposition et les déviations lues au tambour de l'appareil ont été comparées avec une courbe étalon de purpurogalline, obtenue par oxydation du pyrogallol avec du bichromate de potassium.

L'activité a été exprimée en PN (Purpurogallin Number), qui représente la quantité en mg de purpurogalline formée à partir du pyrogallol en 5 min, à 20°, par mg du poids sec de la préparation enzymatique.

Dans nos expériences nous avons utilisé des dilutions (activité comprise entre 3 et 8 PN) de solutions purifiées ayant des activités d'environ 35 P.N.

3. *Ultrasons.* Nous utilisons dans toutes nos études un appareillage à quartz piezoélectrique construit par la maison S.C.A.M.<sup>8</sup>, qui diffère des autres installations par le fait que le faisceau ultrasonore se dégage directement dans l'eau, ce qui présente certains avantages dans l'étude de produits biologiques. Sa puissance ultrasonore est de 76 watts. La fréquence utilisée dans cette étude a été de 960 Kc/sec.

Les solutions d'oxydase (généralement 30 ml) ont été placées dans une cloche en verre, décrite précédemment<sup>1</sup>, dont le fond est constitué par une fine membrane en nitrocellulose, imperméable à l'eau, mais transparente pour les ultrasons. Afin d'éviter l'échauffement du liquide, la cloche a été maintenue pendant l'ultrasonnation dans un bain d'eau glacée.

La phase gazeuse au-dessus de la solution étudiée a été, soit de l'air, soit de l'hydrogène. Ce dernier, débarassé

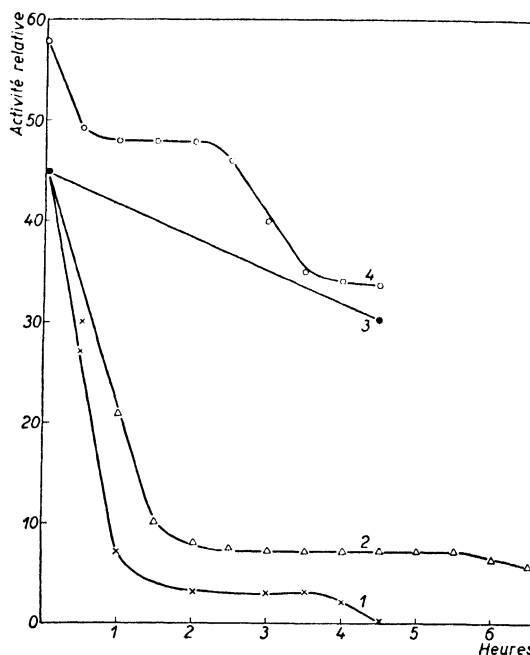


Fig. 1. Activités relatives (les nombres correspondent à des divisions du photomètre) des solutions enzymatiques en fonction du temps d'action: des ultrasons dans l'air (courbe 1); des ultrasons dans l'hydrogène (courbe 2); des ultrasons sans cavitation (3) et de l'agitation avec barbotage d'air (4).



par un barbotage dans une solution de pyrogallol potassique des traces d'oxygène qu'il contient, a été introduit dans la cloche après un dégazage du liquide dans le vide; cette opération a été répétée trois fois pour éliminer l'air aussi complètement que possible.

Des prélèvements du liquide soumis aux ultrasons ont été faits à des temps connus, afin de suivre l'inactivation en fonction du temps. Lorsqu'on travaillait en présence d'hydrogène, on procédait au dégazage et au remplacement de l'air par de l'hydrogène après chaque prélèvement.

Dans l'expérience témoin, où l'on soumettait la solution de l'enzyme à l'action de l'onde ultrasonore sans cavitation, on a utilisé un dispositif spécial, décrit dans un travail antérieur<sup>1</sup>; le liquide préalablement dégazé s'y trouve inclus sans phase gazeuse entre deux membranes dans un récipient cylindrique.

#### RÉSULTATS EXPÉRIMENTAUX

Deux séries d'expériences ont été effectuées en utilisant des solutions d'oxydase ayant des activités différentes. Les résultats ont été similaires. Dans chaque série on soumettait les solutions à l'action des ultrasons, d'une part, en présence d'air et, d'autre part, en présence d'hydrogène, après élimination aussi complète que possible de l'air. Les essais témoins ont été faits, d'une part, en faisant agir les U.S. sur une solution d'oxydase placée dans un récipient cylindrique entièrement rempli de liquide et dégazé, ce qui avait pour but d'éviter l'effet de la cavitation, et d'autre part, en soumettant des solutions d'oxydase soit à une agitation mécanique en présence d'air, soit à un barbotage intense d'air et à une agitation simultanée.

Les résultats obtenus sont représentés par les courbes de la Fig. 1.

#### DISCUSSION

Les solutions enzymatiques employées ne sont pas stables, elles se conservent mal et dans tous nos essais on notait une baisse de l'activité. Mais, si dans les essais témoins (agitation ou action des U.S. sans cavitation) l'inactivation était relativement lente, elle se produisait extrêmement vite sous l'action des U.S. en présence d'air. Ce résultat ne nous étonne pas, puisque de nombreux exemples nous ont montré les effets oxydants des ultrasons et que nous connaissons la sensibilité de l'enzyme employé à des actions oxydantes. Nous soulignerons toutefois la rapidité de l'inactivation. Ce qui est plus étonnant, c'est que l'hydrogène n'ait pas protégé l'enzyme contre les effets des ultrasons; il a cependant ralenti très sensiblement l'inactivation. Or, dans nos expériences antérieures, nous avons généralement constaté que la présence d'hydrogène dans la phase gazeuse empêchait les effets oxydants de se manifester. Jusqu'à présent, nous n'avons observé que deux cas où cette règle ne s'était pas vérifiée: a) l'hydrate manganique brunit, c'est-à-dire s'oxyde en hydrate manganique, même en présence d'hydrogène, bien que beaucoup plus lentement qu'en présence d'air, et b) l'hémagglutinine de *Hemophilus pertussis* est inactivée même en présence d'hydrogène, tandis que la toxine scarlatineuse est protégée dans ces conditions (l'agglutinogène persiste même en présence d'air)<sup>9</sup>.

Si nous croyons avoir proposé une explication plausible du mécanisme des actions oxydantes des U.S.<sup>4</sup>, l'effet de l'hydrogène n'a pas encore été bien expliqué. Il n'est pas impossible qu'il y ait des niveaux différents pour différents accepteurs des produits

oxydants qui se forment dans l'eau sous l'effet des U.S., l'hydrate manganoux étant un accepteur plus puissant que l'hydrogène et ce dernier, à son tour, étant plus actif que le noyau benzénique, etc. Si cette hypothèse est exacte, l'oxydase que nous avons étudiée et l'hémagglutinine de *H. pertussis* auraient une sensibilité voisine de celle de l'hydrate manganoux et on est tenté de se demander si ce n'est pas le composé métallique de l'oxydase qui en est la cause.

Notons que, sous l'effet des U.S., les solutions d'oxydase brunissent et que des essais de réactivation à l'aide du sulfate de cuivre et de la gélatine de l'enzyme<sup>10</sup>, inactivé par les U.S., n'ont pas donné de résultat.

Une autre explication de l'absence de protection par l'hydrogène serait que, dans ce cas, il s'agirait, non pas de l'effet chimique des U.S., mais de l'action mécanique de la cavitation (comme la désintégration des microbes et, probablement, la dégradation des hauts polymères), qui n'est pas influencée par l'hydrogène. Dans l'état actuel de nos connaissances nous ne pouvons pas décider lequel des deux mécanismes connus est en cause ici, mais il nous paraît peu probable qu'il s'agisse d'une action mécanique, étant données les dimensions relativement petites de la molécule d'oxydase.

Avant de conclure, nous voudrions insister sur le fait que nos observations peuvent éventuellement dépendre de notre appareillage, de sa puissance et de la fréquence employée. D'autre part, l'enzyme étudié est particulièrement labile. Il n'est donc pas impossible, et des publications d'autres auteurs le prouvent, d'obtenir à l'aide des U.S. des extraits de cellules contenant des enzymes actifs. Mais ce que nous voulions souligner, c'est que sous l'effet des U.S., certains enzymes peuvent être irréversiblement inactivés et que, par conséquent, si un extrait ne contient pas un enzyme que l'on cherche, cela ne veut pas dire nécessairement qu'il n'existait pas dans la cellule.

### RÉSUMÉ

Les ultrasons (fréquence 960 Kc/sec; intensité 76 watts) inactivent très rapidement des solutions aqueuses de polyphénoloxylase de *Agaricus campestris* purifiée, en présence d'air. Contrairement à ce qui se passe dans de nombreux autres cas, l'hydrogène ne protège pas, mais ralentit seulement cette inactivation, qui est probablement due à l'activité chimique des U.S. Cet exemple montre que l'extraction d'enzymes de cellules à l'aide des U.S. peut être accompagnée éventuellement de leur complète inactivation.

### SUMMARY

Ultrasonic waves (frequency 960 Kc/sec; intensity 76 Watts) very rapidly inactivate in air aqueous solutions of the purified polyphenoloxidase of *Agaricus campestris*. In this case, on contrast to many others, hydrogen does not prevent but only slows down this inactivation probably due to the chemical activity of the ultrasonic waves. This example shows that extraction of enzymes from cells by ultrasonic waves may possibly be accompanied by complete inactivation of the enzymes.

### ZUSAMMENFASSUNG

Ultraschallwellen (Frequenz 960 Kc/Sek; Intensität 76 Watt) inaktivieren an der Luft sehr schnell wässrige Lösungen gereinigter Polyphenoloxylase aus *Agaricus campestris*. Zum Unterschied von vielen anderen Fällen, schützt in diesem Falle Wasserstoff nicht gegen die Inaktivierung, sondern verlangsamt sie nur; diese Inaktivierung scheint durch die chemische Wirkung der Ultraschallwellen bedingt zu werden. Dieses Beispiel lehrt, dass die Extraktion von Enzymen aus Zellen mit Hilfe von Ultraschallwellen gegebenenfalls von einer vollständigen Inaktivierung der Enzyme begleitet sein kann.

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## MICRODOSAGE SPÉCIFIQUE DE L'ACIDE ASPARTIQUE ET DE L'ACIDE GLUTAMIQUE

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On sait que parmi les hydroxyacides qui prennent naissance par action de l'acide nitreux sur les acides aminés actuellement connus comme constituants des protéines, l'acide lactique, l'acide glutamique, l'acide malique et l'acide déhydroxybutyrique, correspondant respectivement à l'alanine, la sérine, l'acide aspartique et la thréonine, fournissent de l'acétaldéhyde par oxydation par le permanganate. FROMAGEOT ET HEITZ<sup>1</sup> ont constaté que cette formation d'acétaldéhyde n'a plus lieu à partir de l'acide glycérique ou de l'acide malique, si ces acides sont en présence d'acétate mercurique; il en est vraisemblablement de même en ce qui concerne l'acide déhydroxybutyrique. Ces réactions constituent la base d'une méthode de dosage spécifique de l'alanine, dans un hydrolysate de protéine, par exemple; mais elles n'avaient pu être utilisées jusqu'ici pour le dosage spécifique de l'acide aspartique, puisque l'acide malique se comporte vis-à-vis de l'acétate mercurique comme les hydroxyacides générateurs d'acétaldéhyde autres que l'acide lactique.

La séparation quantitative par chromatographie, d'après FROMAGEOT, JUTISZ ET LEDERER<sup>2</sup>, des acides aminés en diverses fractions dont une renferme uniquement les acides aminés dicarboxyliques, et une autre uniquement les acides aminés neutres non aromatiques, permet maintenant d'utiliser les réactions précédentes pour le dosage spécifique de l'acide aspartique, et dans un grand nombre de cas, pour celui de l'acide glutamique.

Le *principe* de ces dosages est le suivant: Le mélange des acides aminés, correspondant par exemple à un hydrolysate de protéines, est tout d'abord soumis à la séparation en groupes d'après FROMAGEOT, JUTISZ ET LEDERER<sup>2</sup>; après avoir été ainsi isolés des autres acides aminés, les acides dicarboxyliques sont transformés par l'acide nitreux en hydroxyacides, puis ces derniers sont oxydés par l'acide permanganique; seul alors l'acide aspartique agit en générateur d'acétaldéhyde qu'il est facile de recueillir et de doser spécifiquement. L'acide glutamique est calculé par différence entre l'azote total de la fraction éluée de l'alumine et l'azote de l'acide aspartique.

### MODE OPÉRATOIRE

La *séparation chromatographique* quantitative des acides aminés en groupes a été décrite dans un travail précédent<sup>2</sup>. Nous signalerons seulement ici que la quantité d'alumine "acide" que l'on doit utiliser dépend naturellement de la quantité des acides dicarboxyliques à séparer; il peut être nécessaire d'opérer, par exemple, avec 10 g

d'alumine, au lieu des 5 g dont il est question dans le travail de FROMAGEOT, JUTISZ ET LEDERER<sup>2</sup>, dont les données s'appliquent plus particulièrement à l'insuline.

Les réactions de désamination et d'oxydation se font dans des conditions qui diffèrent de celles indiquées autrefois par FROMAGEOT ET HEITZ<sup>1</sup>, de telle sorte qu'elles permettent de doser des quantités d'acétaldéhyde très inférieures à celles indiquées par ces auteurs. L'appareil utilisé est analogue à celui que ALEXANDER ET SELIGMAN<sup>3</sup> ont décrit pour le dosage de l'alanine; il en diffère par le ballon à réaction, muni d'un rodage normalisé Pyrex no. 2, dont le volume est ici de 100 ml et qui possède une étroite (6 mm de diamètre intérieur) tubulure latérale. Le volume du liquide prélevé sur la fraction des acides dicarboxyliques doit être de 15 ml au maximum. On introduit ce liquide dans le ballon, on ajoute 10 ml d'acide sulfurique 2 N, on porte au bain-marie bouillant, puis, par la tubulure latérale, on introduit goutte à goutte, en agitant vivement au moyen d'un agitateur mécanique 25 ml d'une solution de nitrite de sodium à 5%; la durée de cette introduction doit être de 15 minutes. On rince ensuite la tubulure latérale avec 0.5 à 1 ml d'eau, et on maintient encore 15 minutes le ballon au bain-marie. La désamination est alors terminée. Le ballon étant toujours au bain-marie, on y introduit goutte à goutte, en 15 minutes, toujours en agitant, 7.5 ml d'une solution d'urée à 30%. Il est important que l'acide nitreux soit complètement détruit après cette opération, car des traces de vapeurs nitreuses s'opposeraient à l'utilisation ultérieure du p-hydroxydiphényle pour le dosage colorimétrique de l'acétaldéhyde formé. Après la destruction de l'acide nitreux par l'urée, on rince la tubulure latérale avec 1 ml d'eau, on la ferme, puis on évapore le contenu du ballon sous vide, jusqu'à début de siccité.

On introduit dans le ballon 5 ml d'acide phosphorique 2 N, 1 ml d'une solution de sulfate de manganèse ( $\text{SO}_4\text{Mn}$ ,  $\text{H}_2\text{O}$ ) à 10% et une pincée de talc, puis on le relie à son réfrigérant; on introduit d'autre part dans la tubulure latérale l'extrémité d'une pipette destinée à l'adjonction du permanganate de potassium. Enfin, le tube récepteur, jaugé à 5 ml, est plongé dans un bain de glace; il contient 3 ml d'une solution de bisulfite de sodium à 1%. On provoque une légère dépression dans l'appareil, par aspiration à la trompe, puis on porte le contenu du ballon à ébullition douce, en y introduisant goutte à goutte, en 15 minutes, 10 ml de la solution de permanganate de potassium 0.2 N; on maintient encore l'ébullition pendant 10 minutes; il est important que l'ébullition soit assez douce pour éviter toute condensation soit dans la tubulure latérale, soit dans le tube reliant le réfrigérant au tube récepteur.

Le contenu du tube récepteur est complété à 5 ml, et l'acétaldéhyde recueilli est dosé par colorimétrie, soit par le p-hydroxydiphényle<sup>3</sup> soit par le nitroprussiate<sup>1</sup>, selon les procédés habituels. La méthode au nitroprussiate, moins sensible que celle au p-hydroxydiphényle, a en outre l'inconvénient que la coloration obtenue étant fugace, les lectures photométriques doivent être faites de 3 à 5 minutes après le mélange des réactifs; mais elle a l'avantage sur la méthode au p-hydroxydiphényle d'être moins susceptible à des traces d'impuretés. Parfois, en effet, la coloration obtenue par ce dernier réactif se révèle capricieuse, et fournit, sans cause apparente, un résultat de dosage aberrant.

Le report, sur une courbe obtenue à partir de solutions connues d'acide aspartique, des densités optiques mesurées à partir des solutions à doser, permet de déterminer facilement la teneur en acide aspartique de ces dernières. Les quantités d'acide aspartique, qu'il est ainsi possible de déterminer, sont de l'ordre de 15 à 75  $\mu\text{g}$  quand on utilise la colorimétrie par le p-hydroxydiphényle, et de 0.15 à 2.0 mg, quand on utilise le nitroprussiate.

## RÉSULTATS

Le Tableau I montre les résultats obtenus dans le dosage de quantités connues d'acide aspartique en présence d'acide glutamique.

Les chiffres du Tableau I montrent que, en moyenne, la présence d'acide glutamique abaisse de 5 % le rendement en acétaldéhyde obtenu à partir des solutions ne contenant que de l'acide aspartique. Il convient donc, pour avoir des valeurs aussi exactes que possible, de multiplier les chiffres expérimentaux obtenus par 1.05.

TABLEAU I

DOSAGE DE L'ACIDE ASPARTIQUE DANS DES MÉLANGES CONNUS D'ACIDE ASPARTIQUE ET D'ACIDE GLUTAMIQUE

Acétaldéhyde dosé par le nitroprussiate à l'aide du photomètre de PULFRICH

Composition et volume de la solution des acides aminés dicarboxyliques			Acide aspartique retrouvé	
Aspartique ( $\mu\text{g}$ )	Glutamique ( $\mu\text{g}$ )	Volume (ml)	Valeur absolue	% de la quantité initiale
500	0	2.5	501	100
1 000	0	5.0	1 000	100
500	3 540	7.5	519	104
600	600	6.0	580	97
			600	100
			610	102
600	1 800	12.0	575	96
			535	89
			560	94
600	3 000	15.0	540	90
			600	100
			535	89
1 000	1 416	7.0	930	93
1 000	3 054	10.0	916	92
2 000	703	11.0	1 874	93
Moyenne en présence d'acide glutamique :				95

Le procédé de dosage qui vient d'être décrit a été appliqué d'autre part au dosage des acides dicarboxyliques dans quelques protéines. Les chiffres obtenus sont donnés dans le Tableau II.

Les chiffres du Tableau II appellent les remarques suivantes :

1. L'accord entre les valeurs trouvées pour l'acide aspartique et pour l'acide glutamique dans le présent travail et celles publiées précédemment est tout à fait satisfaisant dans le cas de l'insuline, de l'édénine et de la zéine.

2. Cet accord n'existe pas dans le cas de la gélatine ; mais on doit observer que les valeurs indiquées précédemment pour cette substance ont été obtenues au cours de travaux déjà anciens, et par des procédés dont on sait aujourd'hui qu'ils ne donnent pas de résultats quantitatifs. On peut donc conclure que ce sont les chiffres du présent travail qui correspondent le mieux à la teneur réelle de la gélatine en acide aspartique et acide glutamique.

3. Dans le cas du lysozyme, l'accord est très satisfaisant entre la valeur trouvée pour l'acide aspartique par la présente méthode, et celle que fournit la chromatographie quantitative de partage sur papier d'après FISHER *et coll.*<sup>16</sup>. Mais il n'en est pas de même

TABLEAU II

DOSAGE DE L'ACIDE ASPARTIQUE ET DE L'ACIDE GLUTAMIQUE DANS QUELQUES PROTÉINES.  
ACIDE GLUTAMIQUE CALCULÉ PAR DIFFÉRENCE

Les chiffres ci-dessous sont les moyennes de deux déterminations au moins.

Protéine	N total	N de la fraction dicarboxylique % de la protéine	Acide aspartique trouvé % de la protéine			Acide glutamique trouvé % de la protéine		
			Présent travail	Publié précédemment *		Présent travail	Publié précédemment *	
Gélatine	18.3	1.47	3.1	3.5 9.7	(4) (5)	12.0	6.0 6.2	(4) (5)
Globine (lapin)	16.0	0.59	4.2	—		1.5	—	
Insuline **	15.9	2.39	5.3	5.7 6.8	(6) (7)	19.3	18.6 20.2	(6) (7)
Lysozyme	16.7	2.61	10.2	10.9	(8)	[16.2]	3.0 3.5	(8) (9)
Edestine	18.6	2.73	10.5	12.0 10.1	(10, 11) (12)	16.9	20.6 20.3 19.0	(10) (11) (12)
Zéine	16.0	3.67	3.5	3.2 3.4	(13) (14)	35.0	35.6 30.9	(13) (14)

\* Nous ne donnons ici que les valeurs les plus probables. On en trouvera d'autres dans la bibliographie donnée par BLOCK<sup>15</sup>.

\*\* Insuline cristallisée, à 27 U.I./mg. aimablement offerte par Dr J. LENS, que nous remercions ici bien vivement.

pour l'acide glutamique; il apparaît ainsi que la détermination de cet acide par différence n'est pas possible dans le cas du lysozyme; nous attribuons ce fait à la richesse particulière du lysozyme en tryptophane. La destruction de cet acide aminé au cours de l'hydrolyse par l'acide chlorhydrique entraîne la destruction d'une fraction notable d'autres acides aminés (tyrosine, histidine, etc.) et il est probable que ce sont des fragments azotés résultant de ces destructions, fragments ne réagissant pas à la ninhydrine, qui sont responsables de la richesse en azote de la fraction correspondant à l'éluat de l'alumine "acide".

### RÉSUMÉ

Dans la méthode de dosage proposée, le mélange des acides aminés provenant de l'hydrolyse d'une protéine est d'abord soumis à la séparation en groupes d'après FROMAGEOT, JUTISZ ET LEDERER; après avoir été ainsi isolés des autres acides aminés, les acides dicarboxyliques sont transformés par l'acide nitreux en hydroxyacides, puis ces derniers sont oxydés par l'acide permanganique; seul alors l'acide aspartique agit en générateur d'acétaldéhyde, que l'on dose soit par le p-hydroxydiphényle, soit par le nitroprussiate. L'acide glutamique est calculé par différence entre l'azote total de la fraction élue de l'alumine et l'azote de l'acide aspartique. La méthode permet de doser des quantités d'acide aspartique comprises entre 0.015 et 2.0 mg avec une approximation de 5%. Appliquée à diverses protéines, elle a fourni les résultats suivants: *gélatine*: acide aspartique 3.1, acide glutamique 12.0%; *globine* (lapin): acide aspartique 4.2, acide glutamique 1.5%; *insuline*: acide aspartique 5.3, acide glutamique 19.3%; *lysozyme*: acide aspartique 10.2; *édestine*: acide aspartique 10.5, acide glutamique 16.9%; *zéine*: acide aspartique 3.5, acide glutamique 34.8%. La méthode ne s'applique pas à la détermination de l'acide glutamique dans les protéines riches en tryptophane comme le lysozyme.

### SUMMARY

In the proposed method, the mixture of amino-acids resulting from the hydrolysis of a protein is first separated into groups as described by FROMAGEOT, JUTISZ, AND LEDERER, after isolation from *Bibliographie p. 421.*

the other amino-acids the dicarboxylic acids are converted by nitrous acid into hydroxyacids and the latter are oxidized by permanganic acid; under these conditions, only aspartic acid produces acetaldehyde which is determined quantitatively by p-hydroxydiphenyl or by nitroprussiate. The amount of glutamic acid is calculated from the difference between the total nitrogen content of the eluted fraction and the nitrogen content of the aspartic acid. This method allows the determination of aspartic acid in amounts between 0.015 and 2.0 mg with a maximum error of 5 %. The following results were obtained with different proteins: *gelatin*: aspartic acid 3.1, glutamic acid 12.0 %; *globin* (rabbit): aspartic acid 4.2, glutamic acid 1.5 %; *insuline*: aspartic acid 5.3, glutamic acid 19.3; *lysozyme*: aspartic acid 10.2; *edestin*: aspartic acid 10.5, glutamic acid 16.9; *zein*: aspartic acid 3.5, glutamic acid 34.8. This method cannot be used for the determination of glutamic acid in proteins rich in tryptophan such as lysozyme.

### ZUSAMMENFASSUNG

In der vorgeschlagenen Methode wird die von einem Eiweisshydrolysat herrührende Mischung von Aminosäuren zuerst nach FROMAGEOT, JUTISZ UND LEDERER in Gruppen geteilt; die so isolierten Dicarbonsäuren werden mit salpetriger Säure in Hydroxysäuren verwandelt und dann mit Permanganat oxydiert; nur aus der Asparaginsäure entsteht dann Acetaldehyd und dieses wird mit p-Hydroxydiphenyl oder mit Nitroprussiat bestimmt. Die Differenz zwischen dem Gesamtstickstoff der aus dem Aluminiumoxyd eluierten Fraktion und dem Asparaginsäurestickstoff ergibt den Glutaminsäuregehalt. Mit dieser Methode können 0.015 bis 2.0 mg Asparaginsäure mit einer Genauigkeit von 5 % bestimmt werden. Bei Anwendung auf verschiedene Eiweisstoffe wurden die folgenden Ergebnisse erhalten: *Gelatine*: Asparaginsäure 3.1 %, Glutaminsäure 12.0 %; *Globin* (Kaninchen): Asparaginsäure 4.2 %, Glutaminsäure 1.5 %; *Insulin*: Asparaginsäure 5.3 %, Glutaminsäure 19.3 %; *Lysozym*: Asparaginsäure 10.2 %; *Edestin*: Asparaginsäure 10.5 %, Glutaminsäure 16.9 %; *Zein*: Asparaginsäure 3.5 %, Glutaminsäure 34.8 %. Die Methode kann nicht für die Glutaminsäurebestimmung in tryptophanreichen Eiweisstoffen wie Lysozym angewendet werden.

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## LA NON-RÉVERSIBILITÉ DE LA TRANSFORMATION DE LA MÉTHIONINE OU DE LA THRÉONINE EN ACIDE $\alpha$ -AMINO BUTYRIQUE CHEZ LE RAT

par

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L'acide  $\alpha$ -aminobutyrique n'est pas, comme on l'avait pensé autrefois, un constituant des protéines, mais il se rencontre fréquemment parmi les acides aminés libres dans divers milieux biologiques<sup>1, 2</sup>, en particulier dans l'urine. Les expériences de DENT<sup>1</sup> sur le rat ont montré qu'il se forme à partir de la méthionine. On pouvait alors se demander si la transformation de la méthionine en acide  $\alpha$ -aminobutyrique était réversible et si, par conséquent, il était possible de remplacer la méthionine dans l'alimentation de l'animal, par un mélange convenable d'acide  $\alpha$ -aminobutyrique comme fournisseur de la chaîne carbonée en C<sub>4</sub>, de choline comme donateur du groupement méthyle, et soit de cystine, réductible dans l'organisme en cystéine, soit de sulfure de sodium, comme apport du groupement sulfhydryle. On pouvait également se demander, au cas où la transformation de la méthionine en acide  $\alpha$ -aminobutyrique se révélerait irréversible, si l'apport des substances précédentes à un régime pauvre en méthionine serait suivi d'une économie de cette dernière, autrement dit si cet apport abaisserait la dose minima de méthionine nécessaire à une croissance normale de l'animal.

La possibilité de la réversibilité de la transformation biologique de la méthionine en acide  $\alpha$ -aminobutyrique était rendue vraisemblable par des observations antérieures dont nous avons souligné le caractère préliminaire<sup>3</sup>, mais ces observations, faites dans des conditions matérielles défectueuses, par suite de la destruction du laboratoire, ont dû être reprises. Le présent travail porte ainsi sur l'étude des effets éventuels produits par le remplacement plus ou moins complet de la méthionine, dans un régime auquel sont soumis des rats, par l'acide  $\alpha$ -aminobutyrique accompagné de choline et d'une source de soufre.

D'autre part, des expériences de CHARGAFF ET SPRINSON<sup>4</sup> ont montré la possibilité de la transformation de la thréonine en acide  $\alpha$ -cétobutyrique chez les bactéries et ont rendu vraisemblable l'existence de cette réaction chez les animaux supérieurs; or, on sait que l'acide  $\alpha$ -cétobutyrique peut être facilement transformé chez ces organismes en l'acide aminé correspondant. Aussi avons-nous complété nos investigations par des expériences portant sur le remplacement éventuel de la thréonine par l'acide  $\alpha$ -aminobutyrique, expérience faite dans des conditions analogues à celles portant sur la méthionine.

Disons tout de suite que dans toutes les expériences, conduites cette fois sans aucune ambiguïté, l'acide  $\alpha$ -aminobutyrique s'est constamment révélé incapable de

remplacer, même partiellement, soit la méthionine, soit la thréonine. Il apparaît donc que la transformation de ces acides aminés en acide  $\alpha$ -aminobutyrique est tout à fait irréversible.

## PARTIE EXPÉRIMENTALE

Les animaux utilisés sont de jeunes rats albinos provenant soit de l'élevage de l'INSTITUT PASTEUR, soit de celui de la société L'ALIMENTATION EQUILIBRÉE (rats WISTAR). Au début des expériences, leur poids est compris entre 45 et 75 grammes. Les animaux sont divisés en séries selon la composition des régimes de base auxquels ils sont soumis. Cette composition est indiquée dans le Tableau I.

TABLEAU I  
COMPOSITION DES RÉGIMES DE BASE  
Les chiffres ci-dessous sont exprimés en % du poids du régime total

RÉGIME I		Mélange d'acides aminés* :	
Mélange d'acides aminés . . . . .	20	Pour 100: glycine 0.44; DL-alanine 1.78; DL-	
Saccharose . . . . .	58	valine 8.84; L-Leucine 7.08; DL isoleucine	
Saindoux . . . . .	15	7.97; DL-sérine 0.88; DL-thréonine 6.20; DL-	
Huile de foie de morue . . . . .	3	phénylalanine 6.64; L-tyrosine 4.43; L-tryp-	
Sels minéraux . . . . .	4	tophane 1.79; L-hydroxyproline 0.44; acide	
		L-aspartique 0.88; acide L-glutamique 9.72;	
		L-histidine HCl 3.10; L-arginine HCl 2.66;	
		DL-lysine HCl 19.91; bicarbonate de sodium	
		17.24.	
RÉGIME II		RÉGIME III	
Arachide <sup>3</sup> . . . . .	15	Arachide <sup>3</sup> . . . . .	15
Saccharose . . . . .	40	Saccharose . . . . .	75
Amidon . . . . .	35	Saindoux . . . . .	5
Saindoux . . . . .	5	Huile de foie de morue . . . . .	1
Huile de foie de morue . . . . .	1	Sels minéraux . . . . .	4
Sels minéraux . . . . .	4		

Les sels minéraux sont ceux du mélange de OSBORNE-ET MENDEL<sup>5</sup>.

Les animaux reçoivent en outre une quantité suffisante de l'ensemble des vitamines B, sous forme de "Bécozyme Roche", et un supplément de vitamine A sous forme d'huile de fletan à forte teneur en cette vitamine. Ils disposent de papier filtre et d'eau à volonté.

Chaque animal reçoit chaque jour 10 g de ce régime. On y ajoute les substances dont la nature et la quantité sont indiquées dans les Tableaux II, III et IV, qui donnent le détail des expériences et les résultats obtenus.

Les chiffres de ce tableau permettent les observations suivantes:

1. La présence d'acide  $\alpha$ -aminobutyrique dans un régime ne modifie en aucune façon la croissance d'animaux plus ou moins carencés en méthionine, mais recevant des groupes méthyle et du soufre assimilable en quantités suffisantes, ou en thréonine et recevant d'autre part du soufre assimilable.

2. Le sulfure de sodium s'est montré toxique à la longue dans le cas du régime apportant des acides aminés libres; il a toutefois permis une certaine croissance de deux animaux sur quatre (rats 8 et 10). Au contraire, il a été bien toléré dans le cas des régimes à arachide.

3. Les animaux soumis au régime contenant de l'amidon ont une croissance cons-

\* Ce mélange est celui utilisé par DU VIGNEAUD et ses collaborateurs<sup>6</sup>, sauf en ce qui concerne la proline, qui est remplacée par une quantité égale d'acide glutamique.

tamment supérieure, toutes conditions égales d'ailleurs, à celle des animaux ne recevant comme corps hydrocarboné que du saccharose.

TABLEAU II  
RATS SOUMIS AU RÉGIME I (ACIDES AMINÉS)

T = Durée de l'expérience, en jours

Pi = Poids de l'animal au début de l'expérience, en grammes

Pf = Poids de l'animal à la fin de l'expérience, en grammes

$\Delta P$  = Accroissement moyen journalier du poids, en grammes

C = Consommation moyenne journalière du régime, en grammes

L'acide  $\alpha$ -aminobutyrique est sous la forme DL-.

Rat	Supplément au régime de base (% du régime total)	T	Pi	Pf	$\Delta P$	C
1	{ Méthionine 0.6 Moyenne	32	60	88	0.87	6.7
2			45	64	0.59	7.7
			52.5	76	<u>0.73</u>	7.2
3	{ Méthionine 0.1 Cystine 0.54 Choline 0.50 Moyenne	21	46	58	0.57	6.7
4			72	82	0.47	7.4
			59	70	<u>0.52</u>	7.05
5	{ Méthionine 0.1 Cystine 0.54 Choline 0.50 Ac. $\alpha$ -aminobutyrique 1.08 Moyenne	21	68	77	0.43	6.3
6			58	73	0.71	7.1
			63	75	<u>0.57</u>	6.7
7**	{ Méthionine 0.1 Choline 0.5 Na <sub>2</sub> S.9 H <sub>2</sub> O 0.9 Moyenne	25	54	55	0.57	6.7
8**			75	85	0.40	7.7
			64.5	70	<u>0.22</u>	7.35
9**	{ Méthionine 0.1 Choline 0.5 Na <sub>2</sub> S.9 H <sub>2</sub> O 0.9 Ac. $\alpha$ -aminobutyrique 1.08 Moyenne	13	42	43	0.08	6.2
10**			65	70	0.38	6.9
			53.5	56.5	<u>0.23</u>	6.55
11	{ Choline 0.5 Cystine 0.54 Ac. $\alpha$ -aminobutyrique 1.08 Moyenne	21	49.0	49	0.00	6.2
12			51	49	- 0.10	6.1
			50	49	- <u>0.05</u>	6.15
13	{ Choline 0.5 Cystine 0.54 Moyenne	21	45	44	- 0.05	7.0
14			45	44	- 0.05	6.0
			45	44	- <u>0.05</u>	6.5
15**	{ Méthionine 0.05 Choline 0.5 Cystine 0.54 Moyenne	23	53	65	0.52	6.7
16			64	76	0.52	7.5
			~ 58.5	70.5	<u>0.52</u>	7.1

Rat	Supplément au régime de base (% du régime total)	T	Pi	Pf	$\Delta P$	C
17 18**	Méthionine 0.05 Choline 0.5 Cystine 0.54 Ac. $\alpha$ -aminobutyrique 1.08 Moyenne	22	40 41 40.5	41 43 42	0.05 0.10 <u>0.07</u>	6.7 6.3 6.5
3 bis* 4 bis*	Méthionine 0.6 Cystine 0.54 Moyenne	10	60 82 71	52 71 61.5	- 0.8 - 1.1 <u>- 0.95</u>	4.8 5.2 5.0
5 bis* 6 bis*	Méthionine 0.6 Cystine 0.54 Ac. $\alpha$ -aminobutyrique 1.08 Moyenne	10	79 78 78.5	71 64 67.5	- 0.8 - 1.4 <u>- 1.1</u>	4.8 5.3 5.05

\* Les régimes de ces quatre rats ont été privés de thréonine.

\*\* Rat no. 7 mort le 26ème jour.

Rat no. 8 mort le 31ème jour.

Rat no. 9 mort le 14ème jour.

Rat no. 10 mort le 16ème jour.

Rat no. 15 mort le 24ème jour.

Rat no. 18 mort le 23ème jour.

TABLEAU III  
RATS SOUMIS AU RÉGIME II (ARACHINE, AMIDON)

T = Durée de l'expérience, en jours

Pi = Poids de l'animal au début de l'expérience, en grammes

Pf = Poids de l'animal à la fin de l'expérience, en grammes

$\Delta P$  = Accroissement moyen journalier du poids, en grammes

C = Consommation moyenne journalière du régime, en grammes

L'acide  $\alpha$ -aminobutyrique est sous la forme DL

Rat	Supplément au régime de base (% du régime total)	T	Pi	Pf	$\Delta P$	C
19 20	Méthionine 0.6 Moyenne	46	45 44 44.5	134 130 132	1.94 1.87 <u>1.91</u>	7.58 7.26 7.42
21 22	Choline 0.5 Choline 0.5 Ac. $\alpha$ -aminobutyrique 1.08	46 46	47 45	85 84	<u>0.83</u> <u>0.85</u>	5.94 5.95
23 24	Choline 0.5 Cystine 0.54 Choline 0.5 Cystine 0.54 Ac. $\alpha$ -aminobutyrique 1.08	46 46	45 52	88 87	<u>0.93</u> <u>0.76</u>	5.60 5.53
25 26	Choline 0.5 Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 0.9 Choline 0.5 Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 0.9 Ac. $\alpha$ -aminobutyrique 1.08	46 46	43 47	83 92	<u>0.87</u> <u>0.96</u>	5.80 5.73

TABLEAU IV  
RATS SOUMIS AU RÉGIME III (ARACHINE)

T = Durée de l'expérience, en jours

Pi = Poids de l'animal au début de l'expérience, en grammes

Pf = Poids de l'animal à la fin de l'expérience, en grammes

$\Delta P$  = Accroissement moyen journalier du poids en grammes

C = Consommation moyenne journalière du régime, en grammes

L'acide  $\alpha$ -aminobutyrique est sous la forme DL

Rat	Supplément au régime de base (% du régime total)	T	Pi	Pf	$\Delta P$	C
27	Methionine 0.6	46	40	114	1.61	7.26
68			40	115	1.63	6.43
	Moyenne		40	114.5	1.62	6.85
29	Choline 0.5	46	48	73	0.54	4.83
30			43	70	0.59	5.28
	Moyenne		45.5	71.5	0.57	5.05
31	Choline 0.5	46	42	72	0.72	5.56
	Ac. $\alpha$ -aminobutyrique 1.08					
32	Choline 0.5	46	52	86	0.74	5.86
	Cystine 0.54					
33	Choline 0.5	46	45	84	0.85	5.37
	Cystine 0.54					
	Ac. $\alpha$ -aminobutyrique 1.08					

### RÉSUMÉ

Contrairement à des conclusions publiées précédemment, l'acide  $\alpha$ -aminobutyrique est incapable de remplacer, même partiellement, soit la méthionine, soit la thréonine dans un régime d'autre part complet, sauf en ce qui concerne l'un de ces acides aminés.

### SUMMARY

Contrarily to previously published conclusions, the  $\alpha$ -aminobutyric acid cannot even partially replace methionin or threonin in a diet which is complete except for these two amino acids.

### ZUSAMMENFASSUNG

Im Gegensatz zu früher veröffentlichten Schlussfolgerungen, kann die  $\alpha$ -Aminobuttersäure nicht einmal teilweise Methionin oder Threonin in einer sonst vollständigen Diät ersetzen.

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Reçu le 24 février 1949

## ZUR KENNTNIS DES MECHANISMUS DER ALLOXANWIRKUNG

I. MITTEILUNG:  
DER EINFLUSS VON ALLOXAN AUF EIWEISSTOFFE

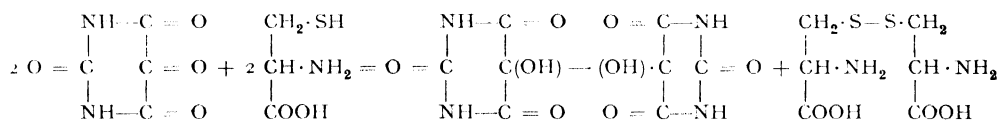
von

ZBYNĚK BRADA

*Forschungsabteilung des Masaryk Radiumtherapeutischen Institutes,  
Brno (Tschechoslowakei)*

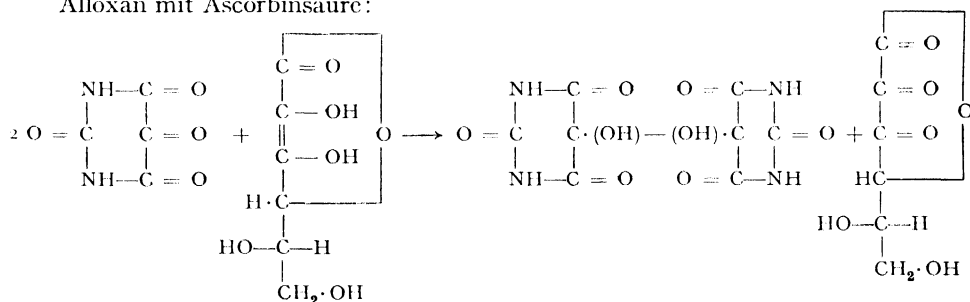
Die Frage der Wirkung des Alloxans bei der Hervorrufung des experimentellen Diabetes wurde schon von manchen Autoren erwähnt<sup>1,2</sup>. Auf Grund der Beobachtungen<sup>3</sup>, dass das Glutathion nach der Alloxaneinspritzung im Blut verschwindet, meint ABDERHALDEN, dass Alloxan nicht direkt wirkt, sondern sofort mit Cystein, Glutathion und Ascorbinsäure reagiert. *In vitro* verlaufen diese Reaktionen auf folgende Weise:

Alloxan mit Cystein:



ABDERHALDEN stellte auch fest, dass Alloxantin diabetogen wirkt, allerdings nicht so intensiv als Alloxan.

Alloxan mit Ascorbinsäure:



Tatsächlich fand auch KARRER<sup>4</sup> und unabhängig von ihm fanden auch LEECH UND BAILEY<sup>5</sup> und TIPSON<sup>6</sup>, im Vergleich zu gesunden Personen, keine Erhöhung der vorhandenen Alloxanmenge in Leichen von Diabetikern. Jedenfalls ist die Menge aber zu gering, um den Diabetes hervorzurufen.

Es gelang ABDERHALDEN und auch anderen Autoren<sup>7</sup> nachzuweisen, dass Tiere die vorher mit Cystein behandelt wurden, gegen die Alloxanwirkung resistent bleiben.

Dieser Befund wurde auch von anderen Autoren bestätigt, die beobachteten, dass durch Fütterung mit einer speziellen Diät, die viel Cystein enthält (Casein usw.) eine analoge Resistenz zu erzielen ist.

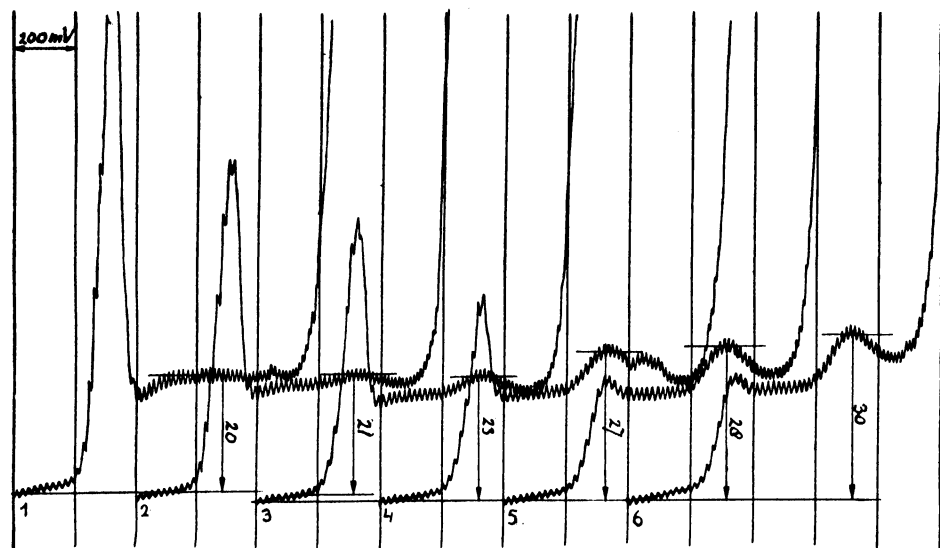


Fig. 1. Kathodische Reduktion der Mischung: /Kobalt (II)-puffer, Alloxan. Empfindlichkeit des Galvanometers 1/300, Akkumulator 4.0 V. Anfang der Reduktion -0.8 V.

Auf Grund dieser Beobachtungen können wir vielleicht auch endlich aufklären, wie es in Literaturangaben<sup>9, 10, 11, 12, 19</sup> über die diabetogene Wirkung der Diallursäure zu manchen Differenzen kam. Durch die Reaktion des Alloxans kommt es nach der Meinung mancher Autoren zur Änderung des Redoxpotentials des Blutes. Dies hat die Zersetzung der  $\beta$ -Zellen der Pankreasdrüse zur Folge. Andere Autoren glauben, dass diese spezifische Wirkung durch eine andere Substanz, die aus Alloxan, oder durch die Alloxanwirkung im Organismus entsteht, verursacht wird.

Die besprochenen Ergebnisse der bisherigen Forschung zwingen uns geradezu nachzuprüfen, ob und in welchem Masse auch Eiweisstoffe durch Alloxanwirkung beeinflusst werden und ob die Wirkung reversibel oder irreversibel ist.

BERNHARD<sup>13</sup> und seine Mitarbeiter versuchten diese Frage zu lösen. Sie prüften ob Alloxan mit dem Bluteiweiss in Reaktion tritt, indem sie den Reststickstoff (RN) im Blut und Serum vor und nach Zugabe bekannter Alloxanmengen bestimmten. Sie stellten fest, dass RN dabei nicht abnimmt, sondern bei höheren Konzentrationen zunimmt.

Die Autoren betrachteten diese Tatsache als Beweis dafür, dass Eiweisskörper durch Alloxan nicht beeinflusst werden.

Die polarographische Methode gibt uns eine Möglichkeit, verschiedene Änderungen in Eiweissmolekülen durch Änderung gewisser physikalisch-chemischer Eigenschaften zu verfolgen.

#### EXPERIMENTELLER TEIL

Der polarographische Teil dieser Arbeit wurde mit dem Polarographen System SHIKATA-HEYROVSKY durchgeführt.

In der ersten Untersuchungsreihe wurden allgemeine polarographische Eigenschaften des Alloxans studiert. Es wurde festgestellt, dass Alloxan unter gewissen Bedingungen polarographisch reduzierbar ist. Die Ergebnisse dieser Versuche werden an anderer Stelle veröffentlicht.

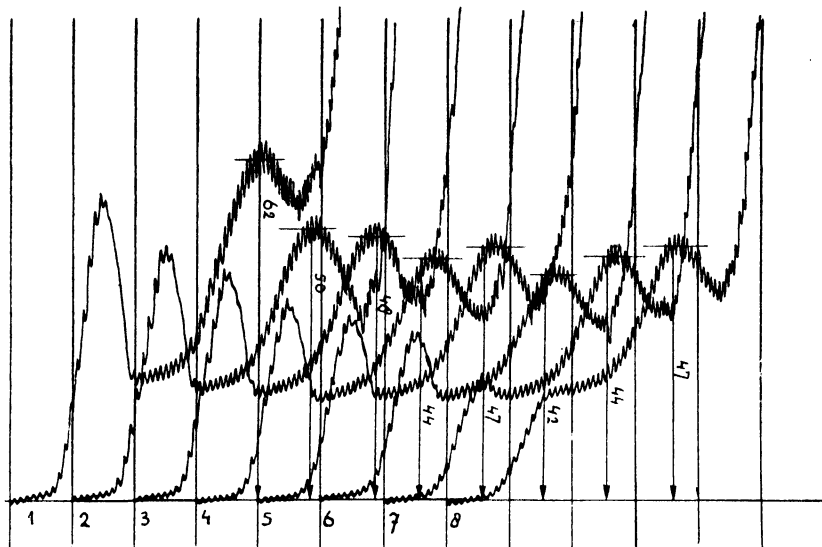


Fig. 2. Polarographische Verfolgung des Einflusses von Alloxan auf die Cystinstufe. Empfindlichkeit des Galvanometers 1/300, Akkumulator 4.0 V. Anfang der Reduktion  $-0.8$  V.

#### EINWIRKUNG VON ALLOXAN AUF KOBALT (II)-PUFFER

Die Zusammensetzung des Puffers war: 1 ml N/100  $\text{CoCl}_2$   
 0.50 ml N  $\text{NH}_4\text{Cl}$   
 0.50 ml N  $\text{NH}_4\text{OH}$

Die untersuchte Lösung wurde hinzugefügt und auf 5 ml aufgefüllt. Siehe Fig. 1 für die erhaltenen Stromspannungskurven.

#### EINFLUSS VON ALLOXAN AUF DIE CYSTINSTUFE

Zur 0.5 ml Cystin M/1000, in n/100 HCl gelöst, wurde 1 ml Alloxanlösung verschiedener Konzentrationen hinzugefügt (siehe bei dem folgenden Versuch die Konzentrationsangaben). Die Mischung wurde eine Stunde stehen gelassen, dann mit Kobalt(II)-puffer vereinigt, auf 5 ml aufgefüllt und polarographiert (Fig. 2).

#### EINFLUSS VON ALLOXAN AUF EIWEISSSTOFFE

##### a) Einfluss auf Ovalbumin

Die Ovalbuminlösung wurde auf folgende Weise dargestellt: 5 g Eialbumin wurden in 100 ml Wasser suspendiert und nach eintägigem Stehen zentrifugiert. Diese Grundlösung wurde dann noch zehnmal verdünnt; also entspricht 1 ml dieser Lösung 0.0025 g Trockensubstanz.

0.25 ml dieser Lösung (0.0006 g Eiweiss entsprechend) wurden in Reagensgläser abpipettiert und dann 1 ml Alloxan in folgenden Konzentrationen hinzugefügt:

Versuchsnummer	Alloxankonzentration	Versuchsnummer	Alloxankonzentration
1	1.00 ml Wasser	5	1.00 ml M/50
2	1.00 ml M/200 All.	6	1.00 ml M/20
3	1.00 ml M/100	7	1.00 ml M/10
4	1.00 ml M/75		

Nach einstündigem Stehen wurden diese Reaktionsgemische mit Kobalt(II)-puffer vermischt und sofort polarographiert (Fig. 3).

##### b) Einfluss von Alloxan auf Blutserum

0.25 ml eines normalen menschlichen Blutserums wurden abpipettiert und 1 ml Alloxanlösung der oben angeführten Konzentrationen hinzugefügt. Nach einer Stunde Stehen wurde diese Lösung zum Kobalt(II)-puffer hinzugefügt. Dann wurde sofort polarographiert. Wir erhielten gleiche Resultate wie mit Eialbumin.

Literatur S. 435.



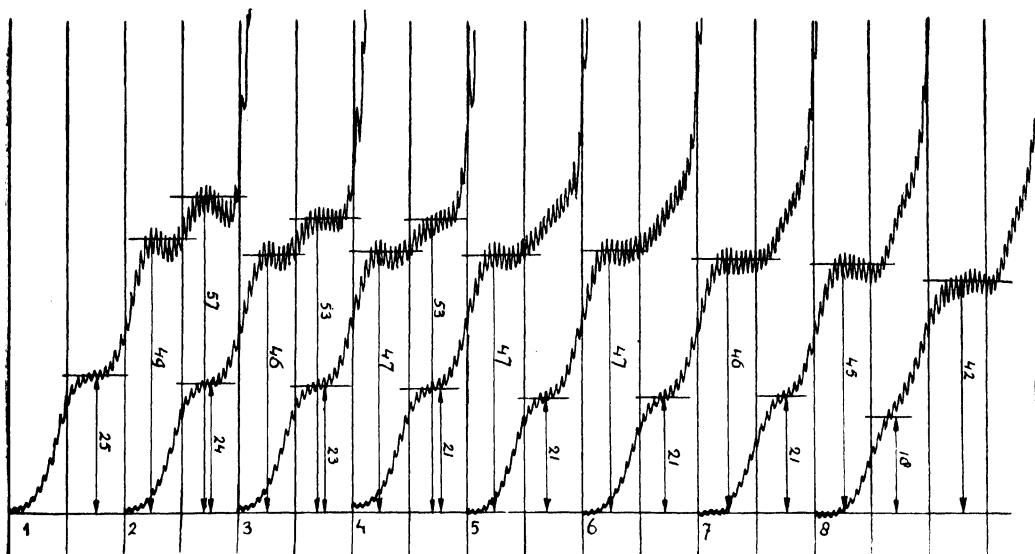


Fig. 3. Polarographische Verfolgung des Einflusses von Alloxan auf Ovalbuminlösung. Akkumulator 4.0 V. Empfindlichkeit des Galvanometers 1/300. Anfang der Reduktion  $-0.8$  V.

#### POLAROGRAPHISCHE EIGENSCHAFTEN DES DURCH ALLOXAN DENATURIERTEN EIWEISSES NACH DIALYSE

Die folgenden Lösungen wurden hergestellt:

- |   |   |  |
|---|---|--|
| a. 2.50 ml Albuminlösung (der oben angeführten Konzentration) | b. 2.50 ml Albuminlösung<br>10.00 ml M/50 Alloxan | c. 2.50 ml Albuminlösung<br>5.00 ml Wasser<br>5.00 ml M/10 Alloxan |
|---|---|--|

Diese Lösungen wurden eine Stunde stehen gelassen und dann 48 Stunden im Dunkeln bei Anwendung von Cellophan als Membrane gegen dest. Wasser dialysiert. Dann wurden die Mischungen auf ein Endvolumen von 12.50 ml aufgefüllt, gut durchgemischt, mit Kobalt(II)-puffer vereinigt und sofort polarographiert.

#### EINFLUSS VON ALLOXAN AUF KOBALT(III)-PUFFER

Darstellung des Puffers: 0.2675 g  $\text{Co}(\text{NH}_3)_6\text{Cl}_3$  75.0 ml 25%  $\text{NH}_3$  (Sp. G. 0.91)  
5.35 g  $\text{NH}_4\text{Cl}$  ad 1000 ml Wasser

Es wurden 4 ml der Pufferlösungen abpipettiert und dann 1 ml der Alloxanlösung folgender Konzentrationen: 1. 1 ml Wasser, 2. 1 M/200, 3. M/100, 4. M/75, 5. M/20, 6. M/10 zugesetzt. Dann wurden die Lösungen sofort polarographiert.

#### DARSTELLUNG VON EIWEISSFREIEN SERUMFILTRATS

Zu 2 ml eines menschlichen Serums wurde 1 ml Alloxanlösung folgender Konzentrationen hinzugefügt: 1. 1 ml Wasser, 2. M/200, 3. M/100, 4. M/75, 5. M/50, 6. M/20, 7. M/10, eine Stunde stehen gelassen und dann 2 ml 20%-iger Sulfosalicylsäure zugegeben. Nach 45 Minuten wurde es zweimal durch dasselbe Filter filtriert.

#### POLAROGRAPHISCHE REDUKTION DES SERUMFILTRATS BEI VERWENDUNG DES KOBALT(III)-PUFFERS

0.3 ml Serumfiltrat wurde mit 4 ml Kobalt(III)-pufferlösung vermischt und von  $-0.8$  V polarographiert.

#### POLAROGRAPHISCHE REDUKTION DES SERUMFILTRATS BEI VERWENDUNG DES KOBALT (II)-PUFFERS

0.3 ml Filtrat wurde mit Kobalt(II)-puffer vereinigt, auf 5 ml aufgefüllt und polarographiert. Siehe Fig. 4.

Literatur S. 435.

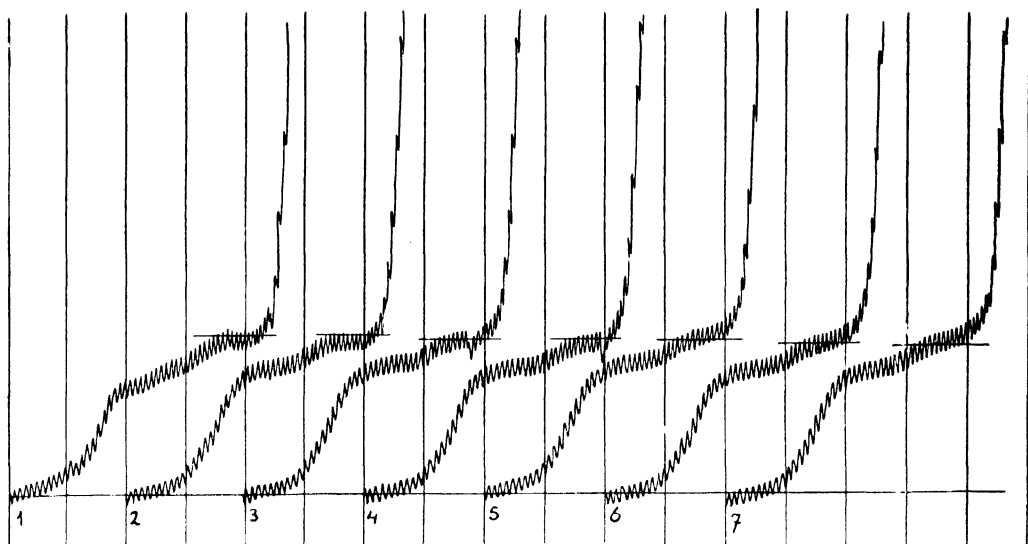


Fig. 4. Polarographische Reduktion des Serumfiltrats (aus einem durch Alloxan behandelten Serum) mit Kobalt(II)-puffer. Akkumulator 4.0 V, Empfindlichkeit des Galvanometers 1/300. Anfang der Reduktion  $-0.8$  V.

#### EINFLUSS VON MONOJODESSIGSÄURE AUF DIE POLAROGRAPHISCHE FILTRATREAKTION MIT DEM KOBALT(II)-PUFFER

Zu 0.3 ml Filtrat wurden 0.20 ml Wasser und 0.20 ml 1%-iger Monojodessigsäure zugegeben. 0.3 ml dieser Lösung wurden mit der Pufferlösung vermischt und polarographiert. Siehe Fig. 5.

#### ÜBER DEN ABBAU DES DURCH ALLOXAN DENATURIERTEN EIERALBUMINS DURCH TRYPSIN

1 g Eieralbumin wurde in eine Reibschale gegeben, dann Alloxan zugefügt, gut durchgemischt, 30 ml Wasser zugegeben und 24 Stunden im Dunkeln stehen gelassen. Nach 24 Stunden wurden 1 ml 10%-iger Lauge und 20 Tropfen Phenolphthalein hinzugefügt. Dann wurde soviel 1 n HCl zugesetzt, um die rote Farbe der Lösung auf rosa zu bringen ( $pH$  ungefähr 8.3). Diese Lösung wurde dann in einem Messkolben auf 100 ml aufgefüllt.

Versuchsnummer	Alloxanmenge	Versuchsnummer	Alloxanmenge
1	25 mg	4	100 „
2	50 „	5	150 „
3	75 „	6	kein Alloxan

0.5 g Trypsin (Merck) wurden in einigen ml destillierten Wassers aufgelöst, mit 10 bis 20 Tropfen Toluol der Lösung im Messkolben beigegeben, welche dann mit destilliertem Wasser auf 100 ml aufgefüllt wird.

Die beiden Lösungen wurden in einen 300 ml Kolben gebracht, in welchem die Lösung noch durchgeschüttelt wurde, um dann der Hydrolyse in einem Wasserbad von  $37^\circ$  unterworfen zu werden.

Die Farbe der Lösung soll nach dem Trypsinzusatz hellrosa sein. Ist dies nicht der Fall, so wird ihr bis zur richtigen Färbung tropfenweise 10%-ige Natronlauge zugegeben. Für die Aufrechterhaltung eines geeigneten  $pH$  musste während der ganzen Dauer der Hydrolyse gesorgt werden. In bestimmten Zeitabschnitten wurden Proben entnommen.

Die Spaltung des Albumins wurde durch das Freiwerden des Aminostickstoffs verfolgt. Die Aminostickstoffbestimmungen wurden nach C. G. POPE UND M. F. STEVENS<sup>36</sup> durchgeführt.

Die Resultate der Blindproben (sofort nach Zusatz von Trypsin) ergaben:

1.	Ohne	Alloxan	0.12 mg	Amino-N	in 100 ml	Hydrolysat
2.	25 „	„	0.56 „	„	„ 100 „	„
3.	50 „	„	0.56 „	„	„ 100 „	„
4.	75 „	„	0.56 „	„	„ 100 „	„
5.	100 „	„	0.89 „	„	„ 100 „	„
6.	150 „	„	1.12 „	„	„ 100 „	„

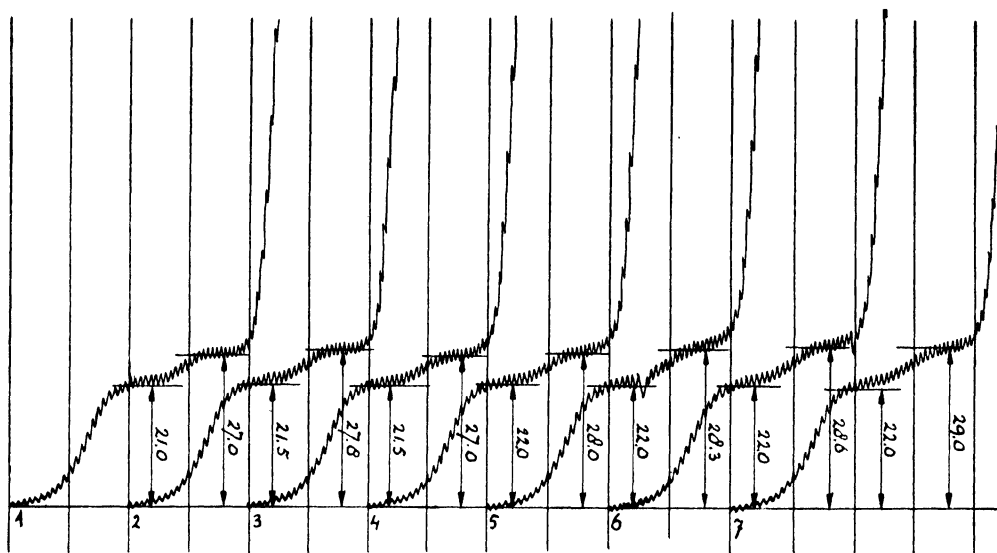


Fig. 5. Einfluss von Monojodessigsäure auf die polarographische Reduktion des Serumfiltrats mit dem Kobalt(II)-puffer. Akkumulator 4.0 V, Empfindlichkeit des Galvanometers  $1/300$ . Anfang der Reduktion  $-0.8$  V.

Proben aus den Hydrolysaten wurden in verschiedenen Zeitabschnitten von 15 Minuten bis 48 Stunden entnommen.

#### ERGEBNISSE UND DISKUSSION

Vor der Untersuchung des Einflusses von Alloxan auf Eiweisstoffe wurde das polarographische Verhalten des Kobaltpuffers und Alloxans im Gemisch geprüft. Es wurde festgestellt, dass Alloxan mit Kobalt(II)-puffer eine Stufe bildet. In der Fig. 6 ist die Abhängigkeit der Stufenhöhe von der Alloxankonzentration graphisch dargestellt.

Das Sauerstoffmaximum wird durch Alloxan, wie das schon früher bei anderen Purinen beobachtet wurde, unterdrückt. Die Kobaltstufe ( $\text{Co}^{\text{II}} \rightarrow \text{Co}$ ) nimmt mit zunehmender Alloxankonzentration ab (Fig. 7).

Dann wurde der Einfluss von Alloxan auf die Cystinstufe der Prüfung untergezogen (Tabelle I).

Die Verminderung der Cystinstufe nimmt bei kleineren Alloxankonzentrationen kontinuierlich ab, später aber, bei grösseren Alloxanmengen nimmt sie unregelmässig zu. Die unregelmässige Steigerung der Cystinstufe bei grösserer Alloxankonzentration als  $M/250$  können wir durch die Bildung der Alloxanstufe erklären (Fig. 8).

In der Tabelle II sind Resultate wiedergegeben, die wir mit durch Alloxan behandelten Eiweisstoffen erhielten.

In der Fig. 9 ist die Abhängigkeit zwischen dem Reduktionspotential der ersten Eiweisstufe und der Alloxankonzentration graphisch dargestellt.

Wir haben also bewiesen, dass die Eiweissdoppelstufe durch Alloxan beeinflusst wird. Nun interessierte es uns zu erfahren, ob wir dasselbe auch bei Serum-eiweisstoffen beobachten könnten. Die Ergebnisse stimmen mit dem Befund, der mit Eialbumin gewonnen wurde, überein.

Um die Frage zu entscheiden, ob die Änderungen, die wir durch Alloxanwirkung auf die Eiweisstoffe erhielten, reversibel oder irreversibel sind, haben wir diese denatu-

rierten Eiweisslösungen gegen destilliertes Wasser dialysiert. Nach der Dialyse sind die Unterschiede im Vergleich mit einer normalen Probe noch deutlicher.

Wir wollten auch untersuchen, wie sich Filtrate, die aus Serum durch Eiweissfällung durch Sulfo-salicylsäure gewonnen wurden, polarographisch verhalten. Die Filtrate polarographierten wir mit Kobalt(II)-puffer und auch mit Kobalt(III)-puffer. Orientationsversuche zeigten, dass die Sauerstoffmaxima in gleicher Weise wie bei dem Puffer II auch bei der Anwendung des Kobalt(III)-puffer beeinflusst werden. Die Höhe der Kobaltstufe ( $\text{Co}^{\text{II}} \rightarrow \text{Co}$ ) bleibt aber unbeeinflusst. In dem Potentialbereich, wo sich die sogenannte Stufe der aktiven Sulfhydrylgruppen bildet, ist eine Erniedrigung zu beobachten. Diese Erniedrigung hat folgende Werte:

Alloxan-konzentration	Stufenhöhe-Änderung in mm
M/200	0
M/100	0
M/75	1
M/20	2
M/10	2

Bei der kathodischen Reduktion des Serumfiltrats mit Kobalt(III)-pufferlösung ist es uns nicht gelungen, Änderungen festzustellen.

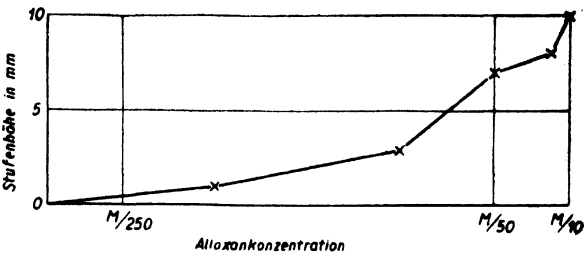


Fig. 6. Zusammenhang zwischen der Stufenhöhe und der Alloxankonzentration im Kobalt(II)-puffergemisch

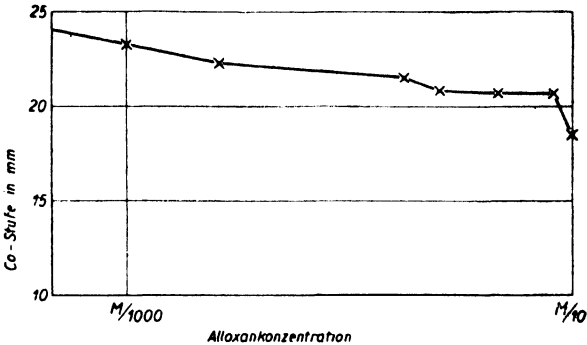


Fig. 7. Abhängigkeit der Höhe der Kobaltstufe von der Alloxankonzentration

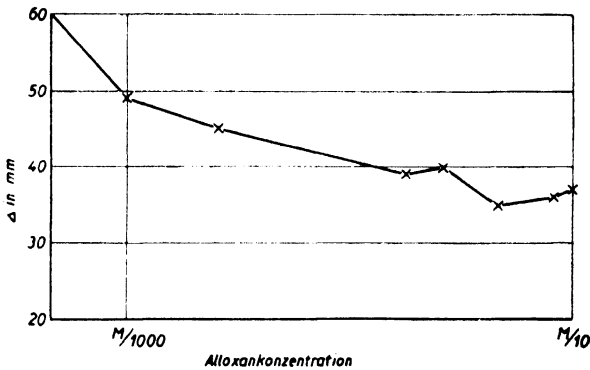


Fig. 8. Zusammenhang zwischen der Differenz Cystinstufe-Alloxanstufe und der Alloxankonzentration

TABELLE I

Nr. der pol. Kurve (Fig. 2)	Alloxankonzentration im Puffergemisch	Höhe der Cystinstufe in mm $h_1$	Höhe der Alloxanstufe ohne Cystin in mm $h_2$	$h_1 - h_2$ in mm	Veränderung der Cystinstufe in mm
1	0	62	—	62	—
2	M/1000	50	1	49	13
3	M/500	48	3	45	17
4	M/375	44	5	39	23
5	M/250	47	7	40	22
6	M/200	42	7	35	27
7	M/100	44	8	36	26
8	M/50	47	10	37	25

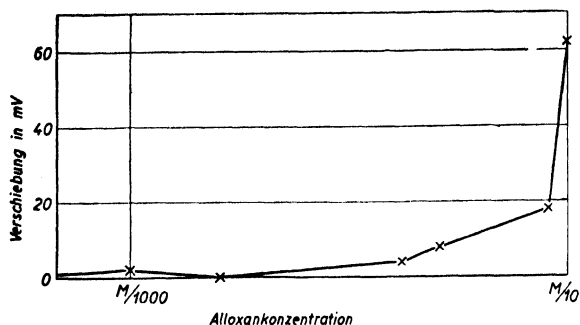


Fig. 9. Einfluss von Alloxan auf das Potential der ersten Eiweisstufe

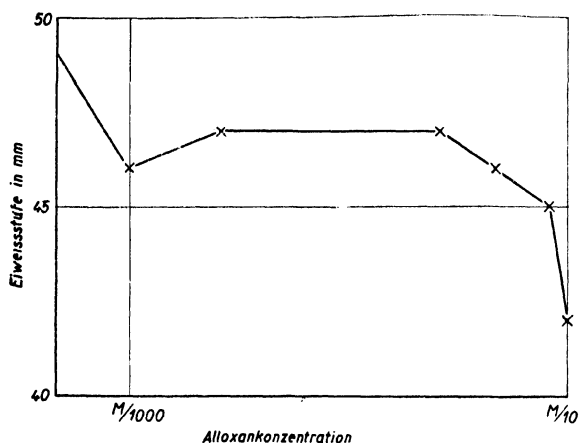


Fig. 10. Einfluss von Alloxan auf die Höhe der ersten Eiweisstufe

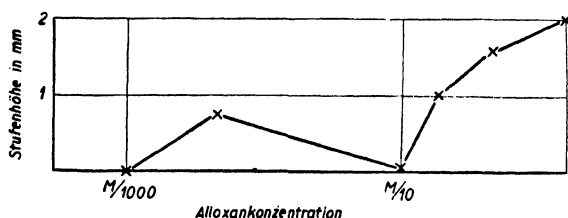


Fig. 11. Abhängigkeit der Stufe der sogenannten aktiven Sulfhydrylgruppen von der Alloxankonzentration bei Anwendung von Monojodessigsäure

Die Verfolgung des Abbaus des Eialbumins durch Trypsin zeigte, dass die Hydrolyse von Eiweiss, das durch Alloxan behandelt wurde, schwerer verläuft. Die Differenzen sind aber nicht sehr gross. Es wurde auch festgestellt, dass Aminostickstoff nach Alloxanzugabe ein wenig zunimmt. Im Vergleich zur zugesetzten Alloxanmenge handelt es sich nur um eine geringfügige Änderung.

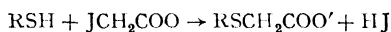
Literatur S. 435.

TABELLE II

Nr. der Kurve (Fig. 3)	Höhe der Eiweissdoppelstufe		Verschiebung des Maximums der ersten Eiweisstufe in V
	S <sub>1</sub>	S <sub>2</sub>	
1	49	57	—
2	46	53	— 0.001
3	47	53	— 0.000
4	47		— 0.005
5	47		— 0.008
6	46		— 0.008
7	45		— 0.018
8	42		— 0.063

Im Gemisch des Filtrates mit Kobalt (II) -pufferlösung beobachteten wir auch keine wesentlichen Änderungen.

Um zu entscheiden, ob es sich um Cystin oder Cystein handelt, lässt man nach R. BRDĚČKA<sup>24</sup> die ammoniakalische Lösung mit Monojodessigsäure reagieren. Die Monojodessigsäure reagiert mit der Cystin SH-Gruppe:



Nach kurzer Zeit verliert Cystein seine polarographische Wirkung, während Cystin seine Reaktionsfähigkeit behält. Wir konnten dabei eine gewisse Erhöhung der Cystinstufe beobachten.

Wir glauben, dass diese Erhöhung mit der Umwandlung von Cystein in Cystin (nach der Auffassung von ABDERHALDEN) zusammenhängt (Siehe Tabelle III).

TABELLE III

Nr. der Kurve (Fig. 5)	Höhe der Stufe in mm
1	27.0
2	27.8
3	27.0
4	28.0
5	28.3
6	28.6
7	29.0

## ZUSAMMENFASSUNG

Es wurde der Einfluss von Alloxan auf Eieralbumin und Blutserum polarographisch verfolgt. Dabei wurde folgendes festgestellt:

1. Die Eiweissdoppelstufe wird durch Alloxan beeinflusst.
2. Die Beeinflussung ist irreversibel, wir können sie durch Dialyse nicht beseitigen.
3. Nach der Beseitigung der polarographischen Stufe von Cystein durch Monojodessigsäure, konnten wir eine Erhöhung der bleibenden Stufe beobachten. Diese Erscheinung schreiben wir dem Cystin zu.

Bei dem Abbau des Eieralbumins durch Trypsin konnten wir bei Alloxanzusatz eine kleine Verminderung der Reaktionsgeschwindigkeit feststellen.

## SUMMARY

The influence of alloxan on egg albumin and blood serum was examined polarographically. The following facts were established:

1. The double wave of protein is influenced by alloxan.
2. The influence is irreversible; it could not be eliminated by dialysis.
3. After elimination of the polarographic wave of cystein by addition of moniodoacetic acid, a rise of the wave was observed. This is attributed to cystine.

During the degradation of egg albumin by trypsin and on addition of alloxan, a small reduction in the reaction velocity was noted.

## RÉSUMÉ

L'étude polarographique de l'influence de l'alloxane sur l'albumine d'œuf et le sérum sanguin montre les faits suivants:

1. L'alloxane modifie la double onde des protéines.
2. Cet effet est irréversible; il ne disparaît pas par dialyse.
3. Après élimination par l'acide moniodoacétique de l'onde polarographique correspondant à la cystéine, l'onde restant s'accroît; ce phénomène doit être attribué à la cystine.

L'alloxane provoque un léger ralentissement de l'hydrolyse de l'albumine d'œuf par la trypsine.

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## RECHERCHES SUR LA PRÉPARATION ET SUR LES PROPRIÉTÉS DE LA THYROGLOBULINE PURE. II

par

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La préparation de thyroglobuline pure au cours d'un précédent travail<sup>1</sup> méritait d'être suivie de recherches sur les propriétés de cette protéine. En effet, les données existant actuellement à ce sujet ont été établies sur des produits plus ou moins impurs et parfois dénaturés; elles présentent, de ce fait, un caractère provisoire et seules celles déterminées par HEIDELBERGER ET SVEDBERG<sup>2</sup>, HEIDELBERGER ET PEDERSEN<sup>3</sup>, LUNDGREN<sup>4</sup> sur l'ultracentrifugation et le point iso-électrique de cette protéine, par CAVETT, RICE ET MAC CLENDON<sup>5</sup> sur sa composition en acides aminés non-iodés, peuvent légitimement être retenues. Quant aux teneurs en iode et en thyroxine de la thyroglobuline dont le rapport (I thyroxinien/I total = 0.3) est à peu près constant<sup>6</sup>, leur variabilité d'une préparation à l'autre<sup>1</sup> pose un problème particulier. Deux hypothèses peuvent être faites pour l'expliquer, soit que le processus d'ioduration de la protéine, physiologiquement plus ou moins intense, s'exerce toujours sur un substrat identique, soit que la teneur en thyroxine de celui-ci et sa structure, variables, régissent la formation de l'hormone. Aussi la composition en acides aminés de la thyroxine de diverses origines devait-elle être étudiée.

Le but de ce travail est de définir: 1. certains caractères physicochimiques de la thyroglobuline pure; 2. la composition en acides aminés de la même protéine extraite de corps thyroïdes normaux ou hypertrophiés de divers mammifères.

### PARTIE EXPÉRIMENTALE

Les thyroglobulines pures étudiées ont été préparées par la méthode décrite dans le premier mémoire de cette série<sup>1</sup>, à partir de lots homogènes (0.25 à 1.50 kg) de corps thyroïdes de Bœuf, de Porc et d'un petit nombre de glandes de Chien. Dans le cas des deux premières espèces, il a été possible de disposer non seulement d'organes normaux (12 à 15 g), mais aussi de glandes fortement hypertrophiées (80 à 115 g). La pureté des produits sur lesquels ont été poursuivies nos recherches a été contrôlée par l'établissement de courbes de solubilité, dont certaines figurent dans notre précédent travail; tous précipitaient entre 36 et 41% de la saturation en sulfate d'ammonium à  $p_H = 6.5$  et à 22° C (concentration en N protéique des essais: 1.5–2.0 mg N/ml).

*Bibliographie p. 441.*

## A. PROPRIÉTÉS PHYSICOCIMIQUES

En dehors de la solubilité des préparations en fonction de la concentration en sels neutres, à  $p_H$  et température constants, nous avons étudié diverses propriétés de la thyroglobuline (Porc, animaux nongoîtreux) afin d'en préciser les caractères. Les données obtenues ont été déterminées à l'Institut de Physicochimie de l'Université d'Upsala au moyen de méthodes aujourd'hui classiques, en sorte qu'il n'y a pas lieu d'en rappeler ici le principe et les modalités d'application\*.

1. *Ultracentrifugation* (SVEDBERG). Les variations de la constante de sédimentation  $s$ , exprimée en unités Svedberg, en fonction de la concentration en protéine, mesurée par la différence de l'indice de réfraction  $\Delta n$  des solutions dialysées contre ClNa 0.2 M et d'une solution de ClNa 0.2 M, sont les suivantes:

$\Delta n \cdot 10^{-5}$	$s_{20}$
0.00427	16.8
0.00073	18.9
0.00037	18.6
0.00018	19.9
0.00009	19.3

La valeur de la constante de sédimentation  $s_{20}$  pour  $\Delta n = 0$ , calculée par extrapolation à la concentration zéro définie à l'aide des données précédentes, est égale à  $s_{20}^0 = 19.4$  S.

2. *Diffusion* (LAMM).  $D_A$  et  $D_M$  ont été respectivement calculées par les méthodes de l'aire et par celle du moment, établies par GRALÉN.

$$D_A = 2.60 \cdot 10^{-7} \text{ cm}^2/\text{sec}$$

$$D_M = 2.61 \cdot 10^{-7} \text{ cm}^2/\text{sec}$$

3. *Volume spécifique partiel*. La détermination de celui-ci, basée sur des mesures picnométriques de densité, a donné les résultats suivants\*\*:

Concentration en protéine (g p. 100 ml)	Volume spécifique partiel, $V_{20}$
1.090	0.721
0.626	0.725

Moyenne: 0.723

4. *Poids moléculaire*. La formule de SVEDBERG, dans laquelle entrent, en dehors des données définies plus haut, la densité du solvant de la protéine  $\rho$ ,  $R$  (constante des gaz) et  $T$  (température absolue) a permis de calculer le poids moléculaire  $M_s$  de la thyroglobuline pure.

$$M_s = \frac{RT \cdot s}{D(1 - V_\rho)} = 653\,000 \text{ (soit } 650\,000)$$

\* L'un de nous (Y.D.) remercie très vivement les Professeurs SVEDBERG ET TISELIUS de l'avoir accueilli dans leurs laboratoires et les fondations (CENTRE NATIONAL DE LA RECHERCHE, FONDATION ROCKEFELLER et FONDATION NOBEL) qui ont bien voulu prendre les dispositions nécessaires pour permettre son séjour à l'Institut de Chimie physique de l'Université d'Upsala.

\*\* Nous remercions le Docteur VIRGIL KOENIG d'avoir procédé à ces déterminations sous la direction du Professeur C. DRÜCKER.



5. *Mobilité électrophorétique* (TISELIUS-SVENSSON). A concentration voisine de 0.5% dans une solution tampon de phosphates alcalins de force ionique 0.2 et de  $p_H = 7.68$ , à 0° et avec un gradient de potentiel de 6.75 volt/cm) la thyroglobuline pure est électrophorétiquement homogène. Sa mobilité exprimée en  $cm^2 \text{ volt}^{-1} \text{ sec}^{-1} \cdot 10^{-5}$  est alors :

$$\begin{aligned} \text{à l'anode: } & 4.62 \cdot 10^{-5} \\ \text{à la cathode: } & 4.46 \cdot 10^{-5} \end{aligned} \quad \text{mobilité moyenne: } 4.54 \cdot 10^{-5}$$

Les valeurs de la constante de sédimentation et du poids moléculaire (déterminé par équilibre de sédimentation) sont voisines de celles obtenues par HEIDELBERGER ET SVEDBERG<sup>2</sup>, HEIDELBERGER ET PEDERSEN<sup>3</sup> sur les thyroglobulines d'Homme et de Porc, à savoir  $s_{20}^0 = 19.2 \text{ S}$  et  $M_s = 650000$ . Les préparations étudiées par HEIDELBERGER ET PEDERSEN renfermaient toujours plus de 20% de constituants de poids moléculaires supérieur et inférieur à cette valeur, la proportion du constituant le plus léger s'élevant notablement dans des milieux très dilués. Il n'en est pas ainsi dans le cas des thyroglobulines pures sur lesquelles nos déterminations ont été effectuées. Cette protéine s'est comportée comme une substance homogène aux diverses dilutions étudiées à l'ultracentrifugation, comme à l'électrophorèse dans les conditions expérimentales indiquées plus haut. Il y a lieu de rappeler que, par contre, l'étude de sa solubilité à  $p_H = 6.50$  a permis d'y déceler trois fractions. Pareil fait a également été observé avec d'autres protéines et nous en poursuivons l'étude.

#### B. COMPOSITION EN ACIDES AMINÉS

Nous nous sommes proposés, d'une part, d'étudier de manière aussi étendue qu'il nous était possible la composition de la thyroglobuline pure de Porc ( $N = 15.8\%$ ) et, d'autre part, de comparer la teneur en certains acides aminés de la même protéine extraite du corps thyroïde de bœufs, de porcs et de chiens normaux ou goitreux (goître simple, de type colloïdal à l'examen histologique). Les résultats figurant dans les Tableaux I et II ont été obtenus par un ensemble de méthodes depuis longtemps éprouvées par deux d'entre nous (R.M. et J.R.)<sup>7</sup> et l'erreur relative qu'ils comportent est en général nettement inférieure à  $\pm 5.0\%$ , sauf dans le cas de la leucine et de la valine, où cette limite est atteinte\*. On les trouvera réunis ci-dessous.

TABLEAU I  
TENEUR EN ACIDES AMINÉS DE LA THYROGLOBULINE PURE DE PORC

Nature de l'acide aminé	p. 100 présent dans la protéine	Nature de l'acide aminé	p. 100 présent dans la protéine	Nature de l'acide aminé	p. 100 présent dans la protéine
Arginine	12.72	Tyrosine	3.12	Alanine	7.40
Histidine	2.23	Diiodotyrosine†	0.54	Glycocolle	3.70
Lysine	3.42	Thyroxine†	0.21	Leucine	12.80
Phénylalanine	6.68	Cystine	3.60	Valine	1.45
Tryptophane	2.08	Méthionine	1.30	Sérine	10.80

† Les teneurs en thyroxine et en diiodotyrosine, de même que celle en iode total ( $I = 0.48\%$ ) n'ont de signification qu'en ce qui concerne la préparation étudiée ici, puisqu'elles sont susceptibles de varier d'un produit à l'autre<sup>1</sup>.

\* Nous remercions le Professeur P. DESNUELLE (Marseille) d'avoir bien voulu doser la méthionine dans la thyroglobuline pure de Porc.

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TABLEAU II

TENEURS EN IODE, EN THYROXINE, EN TYROSINE, EN TRYPTOPHANE ET EN CYSTINE DE THYROGLOBULINES PURES DE DIVERS ANIMAUX NORMAUX OU PORTEURS D'UN GOÛTRE SIMPLE (COLLOÏDAL)

Espèce animale, état et poids des glandes	I total %	Thyroxine %	Tyrosine %	Tryptophane %	Cystine %
Porc, gl. normales (14-16 g)	0.72	0.38	3.15	2.13	3.60
Porc, „ (14-16 g)	0.48	0.21	3.12	2.08	3.55
Porc, „ (14-16 g)	0.47	0.21	3.06	2.11	3.52
Porc, „ (14-16 g)	0.35	0.17	2.12	2.08	3.66
Porc, gl. hypertr. (115 g)	0.04	indosable	3.56	2.08	2.20
Bœuf, gl. normales (14 g)	0.74	0.34	3.55	2.27	3.50
Bœuf, „ (14 g)	0.63	0.29	3.50	2.28	3.55
Bœuf, gl. hypertr. (80 g)	0.01	indosable	5.52	2.25	1.60
Chien, gl. normales*	0.38	non dosée	3.46	2.28	non dosée

\* Poids variant de 1 à 2 g selon celui des animaux.

La teneur en arginine élevée de la thyroglobuline avait été signalée par CAVETT<sup>5, 8</sup>, ECKSTEIN<sup>9</sup>, WHITE<sup>10</sup>; toutefois nos préparations sont sensiblement plus riches en cet acide aminé que celles analysées par eux. Les teneurs en tyrosine et en tryptophane sont voisines dans les produits étudiés par ces auteurs, par BRAND, KASSEL ET HEIDELBERGER<sup>11</sup> et les nôtres. Par contre, des différences se manifestent entre les taux d'histidine et de cystine dans certaines préparations impures et ceux que nous avons déterminés sur le produit pur.

## DISCUSSION

Les données physicochimiques établies sur la thyroglobuline pure n'appellent aucune discussion, étant l'expression de constantes caractéristiques de cette protéine. Par contre, la signification des résultats de nos analyses chimiques doit être dégagée.

Comme nous l'avons montré<sup>1</sup>, les préparations de thyroglobuline pure provenant de lots divers de glandes normales (Bœuf et Porc) sont inégalement riches en iode total, mais présentent une solubilité rigoureusement identique dans des milieux de concentration croissante en sels neutres, en sorte que l'on pouvait penser que leur degré d'halogénéation n'est pas lié à leur composition ou à leur structure, mais à l'activité d'un mécanisme physiologique indépendant de celles-ci. La thyroxine prend naissance par condensation de deux restes de diiodotyrosine provenant de l'halogénéation préalable de la tyrosine. Des écarts importants dans la teneur des préparations en les deux premiers acides aminés, renfermant respectivement 65.4 et 58.7 % I, ne sont pas susceptibles de modifier de manière sensible le taux de tyrosine, car ils portent sur des quantités très faibles de celle-ci dans les glandes normales (0.1-0.2%)\*. La teneur en tyrosine de la thyroglobuline doit donc être relativement constante si le degré d'ioduration de la protéine n'est pas régi par elle. Or, tel est bien le cas et les résultats rassemblés dans le Tableau II montrent qu'il en est de même pour les taux du tryptophane et de la cystéine chez les animaux normaux d'une même espèce. Un ensemble de faits est donc acquis en faveur de l'hypothèse que nous avons antérieurement formulée en ce qui concerne la formation de l'hormone thyroïdienne au sein de la thyroglobuline, à savoir

\* Un enrichissement de 0.200 % en thyroxine (P.M. = 777) est théoriquement compensé par une diminution de 0.093 % du taux de tyrosine (P.M. = 181).

que le corps thyroïde synthétise dans un premier temps cette protéine qui s'halogène par la suite. Protéinogenèse et formation de la diiodotyrosine et de la thyroxine sont des processus entièrement indépendants, le second évoluant sur un substrat toujours identique avec une intensité et une vitesse plus ou moins grandes.

La composition des thyroglobulines ne peut pas être caractérisée par une teneur en iode, mais seulement par le taux des acides aminés non iodés et par des constantes physicochimiques. Celles-ci n'ont été déterminées que dans les préparations de protéines de Porc, tandis que des données chimiques l'ont été sur les thyroglobulines de trois mammifères afin de rechercher si elles traduisent une spécificité analogue à celle des hémoglobines des mêmes animaux<sup>12</sup>. Les différences observées sont assez faibles; toutefois, la thyroglobuline de Porc est moins riche en tyrosine et en tryptophane que celle du Bœuf ou du Chien\*. Enfin, alors que CAVETT, RICE ET MAC CLENDON<sup>5</sup> n'ont pas noté d'écarts nets dans la composition des thyroglobulines d'Homme, provenant de sujets normaux ou goitreux, nous avons mis en évidence une forte diminution de la teneur en cystéine et une augmentation notable du taux de la tyrosine dans les préparations obtenues à partir d'organes hypertrophiés de Bœuf ou de Porc. Le degré de précision de nos méthodes analytiques, supérieur à celui qu'il était possible d'atteindre en 1935, ne doit probablement pas seul être mis en cause et il est possible que les animaux sur lesquels ont porté nos recherches aient présenté un état pathologique particulier. De toute manière, nos observations montrent que la synthèse de la thyroglobuline peut conduire dans des corps thyroïdes hypertrophiés à des protéines de composition anormale. Celles-ci sont, dans les deux cas étudiés, très pauvres en iode, bien que plus riches en tyrosine que les thyroglobulines des animaux normaux, et c'est là une nouvelle preuve que la teneur en tyrosine de la protéine ne commande pas, tout au moins à elle seule, son halogénéation. Quant à leur appauvrissement en cystine, peut-être y a-t-il lieu de le rapprocher du fait que de nombreux "antithyroïdiens" sont des corps sulfurés (thiouracile, sulfocyanures, etc.). Tout se passe donc comme si la formation de la thyroglobuline conduisait dans certains goîtres à une protéine présentant des possibilités accrues d'ioduration, celle-ci étant par ailleurs inhibée. L'étude que nous nous proposons de poursuivre systématiquement sur les thyroglobulines secrétées par des corps thyroïdes présentant des hyperplasies de divers types apportera sans doute de nouveaux éléments à la discussion de ce problème.

### RÉSUMÉ

1. L'ultracentrifugation de la thyroglobuline pure (Porc) a permis de déterminer sa constante de sédimentations ( $s_{20}^0 = 19.4$  S) et son poids moléculaire ( $M_s = 650000$ ). La protéine se comporte comme un corps homogène à l'ultracentrifugation et à l'électrophorèse, mais renferme trois constituants décelables par l'étude de sa solubilité dans des milieux de force ionique élevée.

2. La teneur de la thyroglobuline pure (Porc) en quinze acides aminés a été étudiée.

3. Les thyroglobulines de porcs, de bœufs et de chiens normaux présentent des différences de composition assez faibles. La protéine thyroïdienne de chaque espèce animale est susceptible de renfermer de l'iode à des taux divers, mais sa teneur en tyrosine et en d'autres acides aminés est pratiquement invariable. Il est donc certain que, si la tyrosine est la substance-mère de la thyroxine, son taux ne détermine pas l'intensité de la synthèse de l'hormone au sein de la thyroglobuline. La formation de cette protéine et son ioduration sont deux processus indépendants.

4. Des modifications pathologiques de la composition en acides aminés, en particulier de la teneur en tyrosine et en cystine, ont été enregistrées sur des thyroglobulines d'animaux goitreux.

\* Les données figurant dans les Tableaux I et II ont été établies sur des préparations obtenues à partir d'un grand nombre de glandes, en sorte que chacune d'elles correspond à une moyenne. Les écarts individuels étant, de ce fait, éliminés, les différences observées (dosages en double ou en triple) sont significatives.

## SUMMARY

1. Ultracentrifugation of pure thyroglobulin (pig) has made possible the determination of its sedimentation constant ( $s_{20}^0 = 19.4$  S) and of its molecular weight ( $M_s = 650000$ ). The protein appears to be homogeneous as judged by its behaviour in the ultracentrifuge and during electrophoresis, but contains three constituents as is revealed by its solubility in media of high ionic strength.

2. The content of fifteen amino acids in pure thyroglobulin (pig) has been studied.

3. Thyroglobulins of normal pigs, cattle and dogs show fairly small differences in composition. The thyroid protein of each species can contain varying amounts of iodine, but its content of tyrosine and other amino acids is always the same. It is therefore certain that, tyrosine being the mother-substance of thyroxine, its content does not determine the intensity of the synthesis of the hormone in the thyroglobulin. The formation of this protein and its iodination are two independent processes.

4. In thyroglobulins of goitrous animals pathological changes have been observed in the amino acid composition, especially as regards the content of tyrosine and cystine.

## ZUSAMMENFASSUNG

1. Die Sedimentationskonstante ( $s_{20}^0 = 19.4$  S) und das Molekulargewicht ( $M_s = 650000$ ) von reinem Thyroglobulin (Schwein) wurden durch ultrazentrifugieren bestimmt. Dieser Eiweisstoff verhält sich beim Ultrazentrifugieren und bei der Elektrophorese wie ein homogener Körper, enthält aber drei Bestandteile, die durch Untersuchung der Löslichkeit in stark ionisierten Medien nachgewiesen werden können.

2. Der Gehalt des reinen Thyroglobulins (Schwein) an 15 Aminosäuren wurde untersucht.

3. Die Thyroglobuline von normalen Schweinen, Ochsen oder Hunden zeigen in ihrer Zusammensetzung geringe Unterschiede. Das Schilddrüsen-eiweiß jeder Tierart kann verschiedene Mengen Jod enthalten aber sein Gehalt an Tyrosin und an anderen Aminosäuren ist praktisch konstant. Wenn das Tyrosin auch die Muttersubstanz des Thyroxins ist, so steht es doch fest, dass die Tyrosinmenge die Intensität der Hormonsynthese im Thyroglobulin nicht bestimmt. Die Bildung dieses Proteins und seine Iodierung sind zwei unabhängige Vorgänge.

4. Pathologische Veränderungen der Aminosäurezusammensetzung, insbesondere des Tyrosin- und Cystingehaltes, wurden in den Thyroglobulinen kropfkranker Tiere festgestellt.

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# AU SUJET DES DÉGRADATIONS ET DES SYNTHÈSES EFFECTUÉES PAR *ESCHERICHIA COLI* NON PROLIFÉRANT

par

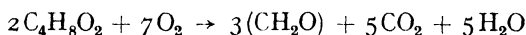
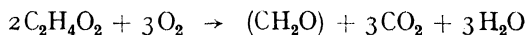
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Un grand nombre de travaux ont été consacrés ces dernières années aux problèmes d'assimilation et de synthèse par les microorganismes hétérotrophes: *Bacterium alcaligenes faecalis*<sup>1</sup>, *Escherichia coli*<sup>1-6</sup>, *Azotobacter vinelandi*<sup>7</sup>, *Pseudomonas calco-acetica*, *Spirillum serpens*<sup>2-6</sup>, algues colorées<sup>8</sup>, levure<sup>9, 10</sup>.

Toutes ces recherches ont été faites en utilisant des méthodes manométriques et des suspensions lavées de microorganismes. Les auteurs ont remarqué que la quantité d'oxygène consommé et la quantité d'anhydride carbonique produit au cours de l'oxydation de différents substrats (tels que lactate, acétate, butyrate, glucose, glycérol) correspondent seulement aux  $\frac{2}{3}$  ou aux  $\frac{3}{4}$  des volumes nécessaires pour une oxydation complète. Ce phénomène serait dû à l'assimilation d'une partie du substrat. Les données obtenues par la consommation d'oxygène, la production d'anhydride carbonique et le quotient respiratoire indiquent que le matériel synthétisé a la formule empirique d'un hydrate de carbone  $(\text{HCHO})_n$ .

Ainsi, pour l'oxydation incomplète d'acide acétique et d'acide butyrique on aura respectivement:



WINZLER ET BAUMBERGER<sup>10</sup>, par des mesures de production de chaleur durant la fermentation alcoolique, ont montré en anaérobiose, un phénomène analogue pour la levure en présence de glucose. VAN NIEL ET ANDERSON<sup>11</sup> ont confirmé ce résultat en dosant l'anhydride carbonique produit au cours de la fermentation alcoolique.

Mais les méthodes manométriques seules n'apportent pas de données suffisantes pour indiquer les produits finaux qui sont formés par oxydation ou fermentation. C'est FROMAGEOT ET SAFAVI<sup>12</sup> qui, en 1939, par l'établissement des bilans des produits de la fermentation, ont montré, à l'aide des bactéries propioniques en croissance, l'existence d'une fermentation liée aux synthèses, foncièrement différente de la fermentation indépendante obtenue à l'aide des masses bactériennes non-proliférantes. En 1943, PICKETT ET CLIFTON<sup>13, 14</sup>, dans un travail sur l'oxydation et l'assimilation par la levure, ont complété leurs expériences manométriques par des dosages chimiques. Ils confirment les résultats déjà obtenus, notamment qu'une suspension lavée de levure "non proliférante" n'est pas capable d'oxyder le glucose complètement.

Ils ont montré, de plus, qu'une partie du substrat (glucose) n'est pas brûlé com-

plètement ni assimilé par la levure, mais converti, au cours de l'oxydation, en produits intermédiaires (tels que glycérol, hexose diphosphate, alcool, acide acétique, acide succinique) non décelables par simple étude manométrique.

L'emploi d'inhibiteurs comme l'azoture de sodium et le 2.4-dinitrophénol permet de confirmer les résultats précédents. Quand ces poisons sont ajoutés à une concentration convenable aux suspensions, on retrouve la quantité théorique d'oxygène et d'anhydride carbonique correspondant à une oxydation complète du substrat. Ainsi, en présence d'azoture de sodium, le quotient respiratoire (QR) atteint la valeur théorique qui était beaucoup plus faible en son absence<sup>2, 3, 5, 6, 15</sup>. L'azoture de sodium à la concentration M/1000, peut inhiber également l'assimilation anaérobie du glucose par la levure sans interférer avec la fermentation<sup>15</sup>. REINER<sup>16</sup> et REINER ET SPIEGELMAN<sup>17</sup> confirment ces expériences. Mais le mécanisme d'action de l'azoture de sodium est encore très obscur, malgré quelques tentatives récentes d'explication<sup>18</sup>.

D'autre part, on n'a, pour toutes les expériences précédentes, aucune idée de la nature des corps synthétisés, en dehors de l'hypothèse, cadrant avec les bilans, d'un corps du type (HCHO). Or, depuis les travaux de WOOD ET WERKMAN<sup>19</sup>, de WERKMAN<sup>20</sup> et de KREBS ET EGGLESTON<sup>21</sup>, on sait que de l'anhydride carbonique peut se fixer sur les corps en C<sub>3</sub>, par suite échapper aux mesures manométriques et participer à des synthèses.

C'est ce qui nous a conduit au présent travail. Nous y montrons l'existence, au cours de la fermentation du glucose ou du pyruvate par une suspension lavée de *E. coli* non proliférante, de synthèse auxquelles participe l'anhydride carbonique, et nous déterminons la nature des produits formés. On verra que, dans nos conditions expérimentales, en outre, ces synthèses peuvent se faire et sont encore plus nettes en présence d'azoture de sodium.

Il faut insister ici, avant de passer à l'exposé des expériences, sur le fait qu'un produit synthétisé n'est pas forcément assimilé. On comprend ainsi que nous ayons pu mettre en évidence des synthèses effectuées par des suspensions bactériennes non proliférantes, sans qu'il s'en suive aucune augmentation de poids sec, ce qui n'était pas le cas dans les expériences de nombreux auteurs.

#### MÉTHODES ET TECHNIQUES

La souche de *E. coli* (souche M) employée provient de l'Institut Pasteur. Elle nous a été donnée par nos collègues J. MONOD ET A. LWOFF, que nous tenons à remercier. Le milieu de culture liquide employé était le suivant : Glucose 1 g, peptone "Byla" 1 g, PO<sub>4</sub>HK<sub>2</sub> 0.2 g, eau distillée pour compléter à 100 ml.

La souche standard est maintenue sur un milieu gélosé contenant les mêmes substances, mais où l'eau est remplacée par de l'extrait de viande de bœuf. Elle est gardée à la glacière.

La suspension bactérienne utilisée dans nos expériences provient de cultures de 16 heures à l'air à 37° sur le milieu liquide cité\*. (Pour 100 ml de milieu on a environ 25 mg de poids sec). Après centrifugation, les bactéries sont lavées une fois avec du chlorure de sodium à 0.9%, puis remises en suspension dans la solution de chlorure de sodium. On y fait passer, comme l'a recommandé QUASTEL, pendant 3/4 d'heure, à l'abri de la lumière, un courant d'air pour brûler les réserves endogènes. Dans les expériences avec inhibiteur, celui-ci est ajouté à la suspension juste avant le passage de l'air. On a ainsi un contact préalable de la suspension avec l'inhibiteur qui réduit les erreurs éventuelles dues à la perméabilité. On recentrifuge ensuite les bactéries et on les met en suspension dans un tampon de phosphate M/15, pH 6.8. Cette suspension est faite immédiatement avant chaque expérience.

\* On ensemence toujours largement (2 ml pour 100 ml) à partir de cultures âgées de 6 heures, en milieu liquide de même composition.

Chaque ballon d'expérience contient: la suspension bactérienne (5 ml) que l'on ajoute au tampon phosphate M/15  $p_H$  6.8 et au substrat, stérilisé à part dans l'eau distillée et neutralisée, s'il y a lieu. Le volume total est de 100 ml. Les conditions d'asepsie sont strictement observées au cours des différentes opérations.

Les ballons sont mis à l'étuve à 37° pendant 22 heures. Au bout de ce temps on réajuste à 100 ml.

Les conditions d'aérobiose ou d'anaérobiose ont été observées très strictement. L'aérobiose était assurée, soit par passage d'air stérile, soit par agitation dans le thermostat à 37°. Si on laisse simplement le milieu avec la suspension bactérienne à l'étuve sans agitation, il s'établit très vite des conditions de semi-anaérobiose, faussant complètement les résultats. Dans le cas de l'anaérobiose, le milieu est désaéré préalablement par ébullition à l'autoclave ouvert et refroidi, sans agitation. Le vide est fait au moyen d'une pompe à huile, qui donne une pression d'air résiduel de 0.1 mm de mercure. Il reste en général, dans nos milieux, de 0.9 à 1.8 ml d'azote pour un volume de 300 ml du ballon. Nous avons remarqué que le métabolisme varie d'une façon très sensible si on laisse dans le milieu plus de 3 à 5% d'air.

Afin de saisir les produits intermédiaires, nous n'avons jamais travaillé jusqu'à épuisement du substrat, qui est toujours en léger excès.

La densité bactérienne a été évaluée par opacimétrie, à l'électrophotomètre de Meunier avec écran bleu; on prélevait 2 ml de culture que l'on complétait à 5 ml avec de l'eau distillée. Parfois aussi, et à titre de recoupement, on a déterminé le poids sec des bactéries mises en expériences: 20 mg correspondent à une "opacité" de 112 divisions au photomètre.

Pour les dosages, le milieu est centrifugé à 13000 tours pendant 15 minutes; les prélèvements sont faits sur le liquide surnageant.

Naturellement, l'opacité et les dosages initiaux ont été effectués à part, dans les mêmes conditions.

Les sucres réducteurs ont été dosés par la méthode de HAGEDORN ET JENSEN<sup>22</sup>; l'acide lactique par celle de FRIEDEMANN<sup>23</sup>, à l'aide de l'appareil de FUCHS<sup>24</sup>; l'acide pyruvique par celle de WARBURG<sup>25, 26</sup> à la carboxylase; l'acide succinique et l'acide fumarique par celle de KREBS<sup>27, 28</sup> à la succinodéshydrogénase.

L'alcool éthylique est dosé par la méthode de NICLOUX<sup>29</sup>. On prélève une prise distincte de la solution à doser et on fait, dans un appareil rodé, deux distillations successives pour éliminer toutes substances réductrices gênant le dosage. On distille tout d'abord en milieu acide les  $\frac{3}{4}$  de la prise, puis le distillat est complété au volume de la prise initiale avec de l'eau bidistillée bouillie, et on redistille à nouveau les  $\frac{3}{4}$  du volume, en milieu alcalin. Le dosage est fait sur ce dernier distillat.

Le dosage des acides volatils est fait sur une prise distincte, dans un appareil rodé, par entraînement à la vapeur d'eau. La distillation est faite en atmosphère d'azote pour éviter la formation et l'entraînement de traces d'acides volatils provenant de l'oxydation d'impuretés présentes dans le milieu. Cette précaution s'est avérée indispensable. Sur le distillat ainsi obtenu, on dose l'acidité volatile totale, en prenant la précaution d'éliminer l'anhydride carbonique présent. L'acide formique est dosé par la méthode au calomel<sup>30</sup>; l'acide acétique est obtenu par différence. Le dosage de l'anhydride carbonique a été fait de la manière suivante:

**Aérobiose:** L'anhydride carbonique dégagé au cours de l'expérience est déplacé par un courant d'air privé d'anhydride carbonique, et recueilli dans la soude à 10% décarbonatée; il est ensuite dosé par la méthode de DENIGÈS<sup>31</sup>.

**Anaérobiose:** a) Dans certaines expériences, on a voulu absorber l'anhydride carbonique produit au cours de la fermentation. Dans ce but, on a soudé au fond du ballon une cupule de 6 cm de haut et de 2 cm de diamètre environ. 2 ml de soude décarbonatée à 10% sont ajoutés dans cette cupule juste avant le vide. A la fin de l'expérience, on prélève quantitativement la soude dans une fiole jaugée. Le dosage est fait sur une prise par la méthode de DENIGÈS<sup>31</sup>. b) Dans toutes les autres

\* La rétention éventuelle de l'anhydride carbonique dans le milieu est mesurée sur une prise, dans l'appareil de Warburg, par déplacement avec de l'acide sulfurique 5 N.

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expériences, le dosage était fait par extraction des gaz totaux. Une analyse préliminaire par absorption du mélange du gaz sur gel de silice refroidi à  $-192^{\circ}$ , a montré que la teneur en méthane était inférieure à 1 %; la séparation des constituants de ce mélange par l'air liquide ne se justifiait donc pas. La technique utilisée a été la suivante: 1. Le ballon d'expérience est fixé par son rodage à une trompe à mercure sans joints de nature organique (trompe Delaplace); on fait le vide complet, on ouvre ensuite le robinet et on extrait la totalité des gaz. Ces gaz, recueillis dans une éprouvette, sont mesurés sur la cuve à mercure. 2. Après la mesure du volume total extrait, on laisse les gaz en contact avec quelques pastilles de potasse, jusqu'à volume constant, pour absorber la totalité de l'anhydride carbonique. La différence des valeurs mesurées donne l'anhydride carbonique total. 3. On prend alors 1 ml environ du gaz restant, mesuré dans des jauges de précision au centième (lecture au contact) et on procède comme suit à l'analyse:

Les traces d'oxygène libre sont mesurées préalablement par absorption au pyrogallate de potasse faite *in situ* dans un tube à essais qui sert sur la cuve à mercure de tube d'absorption. Le gaz restant est alors analysé par la technique classique eudiométrique, l'oxygène ajouté étant préparé par décomposition dans le vide du permanganate de potassium et passage dans l'air liquide. Le gaz résiduel donne l'azote. La méthode est extrêmement précise: les erreurs sont de l'ordre de 0.2-0.3 %.

Nous avons travaillé, comme il a été dit précédemment, avec des bactéries non proliférantes; il n'y avait donc aucune augmentation de poids, mais plutôt, au contraire, légère autolyse. Pour réduire au maximum les causes d'erreurs dues à l'autolyse des bactéries, nous avons essayé de réduire le temps des expériences. Au bout des 6 premières heures, il ne se produit aucune autolyse en présence du substrat, mais dans ce laps de temps la consommation en substrat est faible et les erreurs qu'on peut faire sur les dosages des petites quantités de produits formés peuvent s'accumuler et devenir non négligeables. Par contre, au bout de 22 heures, la consommation du substrat atteint environ 150 mg et l'autolyse est faible. Pour éliminer au maximum les erreurs dues même à une faible autolyse, nous avons fait des expériences témoins. Celles-ci étaient réalisées, au début, dans les mêmes conditions expérimentales, mais sans substrat. Mais dans ce cas là, l'autolyse est nettement plus forte que dans les expériences avec substrat. Elle se produit déjà 2 heures après la mise en route et on obtient une très forte quantité d'acide acétique et d'alcool dont l'origine reste pour le moment inexpliquée. Ainsi, sans substrat, le métabolisme bactérien est complètement changé; ce n'est donc plus une véritable expérience témoin. Nous avons été amenés à faire ces témoins en laissant les bactéries dans le tampon phosphate  $p_H$  6.8 simplement pendant une heure. A ce moment, la densité optique est sensiblement la même qu'à la fin des expériences. Les dosages des témoins sont faits, après centrifugation, sur le liquide surnageant, et les chiffres trouvés sont retranchés de ceux de l'expérience.

Nous avons exposé en détail les techniques que nous avons employées, car les bilans peuvent varier d'expériences en expériences suivant la souche, la quantité de bactéries, la quantité de substrat utilisé, la durée des expériences, le degré d'aérobiose ou d'anaérobiose. Mais par une standardisation stricte des conditions expérimentales, on arrive à avoir des bilans relativement très constants.

#### RÉSULTATS EXPÉRIMENTAUX

Dans les premières expériences, on a étudié les produits obtenus lors de la fermentation du glucose dans les conditions normales énoncées précédemment. Le dosage des gaz a été fait par extraction. Les bilans obtenus sont donnés dans le Tableau I, en mg et en % de carbone.

Suivant le schéma classique de HARDEN<sup>32</sup>, on devrait trouver une quantité équimoléculaire de corps en  $C_1$  et de corps en  $C_2$ :



TABLEAU I  
ACTION DE *E. COLI* SUR LE GLUCOSE EN ANAÉROBIOSE

Concentration du glucose 0.02 M  
Tous les chiffres sont calculés en carbone

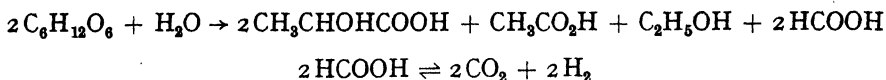
Expérience:	a		b		c*		d*		e	
	mg	%	mg	%	mg	%	mg	%	mg	%
Opacité: initiale	112		75		59		63		100	
finale	94		56		30		39		85	
Glucose disparu (mg)	48.6		41		26		34		67.4	
Produits formés	mg	%	mg	%	mg	%	mg	%	mg	%
Acide succinique	2.3	4.8	3.0	7.3	0.75	2.9	traces		4.95	7.35
Acide lactique	8.8	18.1	10.9	26.5	4.63	17.8	7.46	21.98	13.9	20.6
Acide pyruvique	1.2	2.5	1.5	3.7	0.78	3	traces		0.71	1.06
Alcool	10.7	22	6.6	16.2	4.85	18.65	9.5	27.98	14.72	21.9
Acide acétique	20.8	41.5	12.4	30.4	12.3	47.23	13.05	38.2	21.2	31.2
Acide formique	traces		1.0	2.5	1.11	4.26	0.82	2.44	1.78	2.68
Anhydride carbonique	4.9	10.1	3.7	9.0	2.6	7.95	3.93	11.55	7.8	11.6
Méthane	0.05	0.1	0.03	0.08	0	0	0	0	0	0
Carbone retrouvé	—	99.1	—	95.8	—	101.79	—	102.15	—	96.39
Hydrogène en ml pour 100 mg de carbone du glucose disparu		21.4		21.4		—		26.6		21.5
Azote résiduel (ml)		1.23		0.81		—		1.17		1.14
Oxygène résiduel (ml)		0		0		—		0.58		0

\* Autolyse notable

TABEAU II  
ACTION DE *E. COLI* SUR LE PYRUVATE DE SODIUM EN ANAÉROBIOSE  
Concentration du pyruvate 0.025  
Tous les chiffres sont calculés en carbone

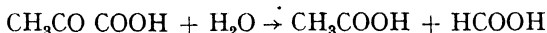
Expérience :	f		g*		h*		i*		k	
	mg	%	mg	%	mg	%	mg	%	mg	%
Opacité: initiale		110		83		94.5		94.5		165
finale		98		61		73		73		149
Pyruvate disparu (mg)		79.2		77.7		67.4		67.4		72
Produits formés										
Acide succinique	1.43	1.8	3.9	5	traces		traces		1.01	1.4
Acide lactique	1.39	1.75	2.61	3.4	1.49	2.2	1.49	2.2	3.45	4.8
Alcools	0	0	0	0	0	0	0	0	0	0
Acide acétique	57.6	72.8	58.2	74.6	53.6	79.5	52.4	77.7	53	73.7
Acide formique	8.55	11	7.8	10	5.05	7.47	7.12	10.5	5.25	7.8
Anhydride carbonique	9.85	12.45	7.765	10	10.5	15.5	9.28	13.7	9.86	13.7
										21.5
Carboné retrouvé	—	99.8	—	103	—	104.67	—	104.1	—	101.4
Hydrogène en ml pour 100 mg de carbone du glucose disparu		non dosé		non dosé		32.4		29.6		non dosé
Azote résiduel (ml)		1.32						1.32		
Oxygène résiduel (ml)								0.24		

\* Autolyse notable



ce qui donne deux atomes de carbone correspondant à l'anhydride carbonique pour quatre atomes de carbone correspondant à l'alcool et à l'acide acétique.

On sait, d'autre part, que l'origine de l'acide acétique est expliquée par la réaction:



Or, ceci ne correspond pas du tout aux chiffres du Tableau I. Dans l'expérience *a* par exemple, on devrait, pour 63.5 de C de corps en  $\text{C}_2$ , avoir  $63.5 : 2 = 31.75$  de C de corps en  $\text{C}_1$ ; or, on en obtient 10.2, soit 36% environ de la valeur calculée. En faisant le même calcul pour les autres expériences, on ne retrouve que: 58.5% (expérience *b*), 39% (expérience *c*), 42.3% (expérience *d*), 61% (expérience *e*).

On voit donc qu'il y a toujours un grand écart entre les valeurs théoriques d'anhydride carbonique calculées par rapport aux corps en  $\text{C}_2$  et les valeurs trouvées.

Nous avons répété les mêmes expériences en prenant le pyruvate comme substrat. Les résultats sont donnés par le Tableau II. Les calculs précédents pour le rapport *anhydride carbonique trouvé/anhydride carbonique théorique* (l'anhydride carbonique théorique étant égal à 2 (*somme des corps en  $\text{C}_1$ /somme des corps en  $\text{C}_2$* )) donnent: 65.6% (expérience *f*), 57% (expérience *g*), 58% (expérience *h*), 62% (expérience *i*), 59% (expérience *k*).

Ici, l'écart que nous avons remarqué avec le glucose entre l'anhydride carbonique trouvé et l'anhydride carbonique théorique, se produit également, mais le pourcentage d'anhydride carbonique trouvé par rapport à la valeur théorique est plus fort que lorsqu'on ajoute du glucose comme substrat.

L'examen des bilans montre donc un déficit d'anhydride carbonique. On y constate aussi un déficit parallèle d'hydrogène. Il convient de rechercher la signification de ce phénomène.

Puisque, suivant SPIEGELMAN<sup>18</sup>, CLIFTON<sup>2, 3</sup> et WINZLER<sup>15</sup>, l'azoture de sodium empêche l'assimilation et les synthèses, il était logique de refaire ces mêmes expériences en présence d'azoture de sodium. Les résultats sont donnés dans le Tableau III.

Les résultats de ce tableau montrent la diminution de l'anhydride carbonique et de l'hydrogène en présence d'azoture de sodium, ce qui confirme les résultats déjà obtenus par différents auteurs\*. La diminution de l'anhydride carbonique (expérience *m*, Tableau III) par rapport aux bactéries sans azoture de sodium (expérience *e*, Tableau I) est de 78% et celle de l'hydrogène de 99%. Les expériences *b* et *c* du Tableau I et l'expérience *m* du Tableau III sont faites dans les mêmes conditions avec la même suspension. La diminution des deux gaz est du même ordre dans les différents bilans, même si on abaisse la concentration en azoture de sodium à M/800 et M/1600. Mais il ne faut pas confondre la diminution de l'anhydride carbonique et de l'hydrogène avec l'inhibition de la fermentation. En présence d'azoture de sodium, la fermentation est caractérisée par une augmentation d'acide lactique et, d'autre part, nous voyons que le déficit en anhydride carbonique est beaucoup plus grand qu'en l'absence d'azoture. En effet, le pourcentage de l'anhydride carbonique trouvé par rapport à l'anhydride carbonique théorique, est, dans les deux expériences du Tableau III, de 15%. Ces valeurs

\* Remarquons aussi que l'azoture de sodium diminue l'autolyse; il doit agir, probablement, sur les ferments protéolytiques.

TABLEAU III

ACTION DE *E. COLI* SUR LE GLUCOSE EN ANAÉROBIOSE ET EN PRÉSENCE D'AZOTURE DE SODIUM  
 Concentration de l'azoture de sodium  
 Concentration du glucose 0.02 M  
 Tous les chiffres sont calculés en carbone

Expérience :	l		m	
Opacité: initiale	119		100	
finale	112		96	
Glucose disparu (mg)	87.4		55.4	
Produits formés	mg	%	mg	%
Ac. succinique . . . . .	traces		0.29	0.54
Ac. lactique . . . . .	53	60.6	34.9	63
Ac. pyruvique . . . . .	2.54	2.9	1.09	1.97
Alcools . . . . .	10.7	12.3	5.57	10.05
Ac. acétique . . . . .	22.4	25.6	14.28	25.8
Ac. formique . . . . .	1.1	1.26	traces	
Anhydride carbonique . . . . .	1.38	1.58	1.435	2.6
Méthane . . . . .	0	0	0	0
Carbone retrouvé . . . . .	—	103.64	—	103.96
Hydrogène en ml pour 100 mg de carbone du glucose disparu		0.64		1.9
Azote résiduel (ml) . . . . .		non dosé		0.42
Oxygène résiduel (ml) . . . . .		non dosé		0.06

sont beaucoup plus faibles que celles que l'on observe dans les expériences sans azoture de sodium\*.

Les mêmes expériences ont été répétées en remplaçant le glucose par le pyruvate. Mais ce dernier est mal attaqué en anaérobiose en présence d'azoture de sodium; l'autolyse était très forte, ce qui faussait les bilans.

Nous avons refait les mêmes expériences à l'air. Les résultats sont donnés dans le Tableau IV.

D'après les chiffres, nous voyons qu'à l'air, en présence ou en l'absence d'azoture, avec le glucose ou le pyruvate comme substrat, le pourcentage d'anhydride carbonique par rapport aux corps en C<sub>2</sub> est, aux erreurs d'expérience près, celui qu'on devrait obtenir théoriquement. D'autre part, à l'air, il y a une augmentation d'acide pyruvique en présence d'azoture et la consommation du substrat est toujours augmentée.

L'ensemble des résultats des expériences précédentes s'explique si l'on admet 1. qu'en anaérobiose, en présence ou en absence d'azoture de sodium, une partie du gaz carbonique et de l'hydrogène est réutilisée pour des synthèses, tandis qu'à l'air ces synthèses sont supprimées dans les deux cas; 2. que l'azoture de sodium n'inhibe pas plus la respiration que la fermentation de *E. coli*; dans le premier cas, il y a augmentation d'acide pyruvique, dans le second cas, d'acide lactique.

Il reste donc à savoir ce que devient l'anhydride carbonique en anaérobiose. La première idée qui vient à l'esprit, c'est que, suivant WERKMAN ET WOOD<sup>19, 20</sup>, l'anhydride carbonique se combine à un corps en C<sub>3</sub> en donnant l'acide succinique qui, à son

\* Nous nous sommes assurés qu'il ne s'agit pas d'une mutation, ni d'une adaptation de *E. coli* à l'azoture de sodium, en repiquant les bactéries sur un milieu contenant l'azoture.

TABLEAU IV

ACTIONS COMPARÉES DE *E. COLI* SUR LE GLUCOSE ET LE PYRUVATE EN AÉROBIOSE, EN PRÉSENCE ET EN ABSENCE D'AZOTURE DE SODIUM

Concentration du glucose 0.02 M

Concentration du pyruvate 0.025 M

Concentration de l'azoture de sodium 0.003 M

Tous les chiffres sont calculés en carbone

Expérience :	n glucose seul		o glucose + azoture		p pyruvate seul		q pyruvate + azoture	
Opacité: initiale	111.5		111.5		115.5		115.5	
finale	93		97		107		104	
Substrat disparu (mg)	70.6		121.2		43.8		71.5	
Produits formés	mg	%	mg	%	mg	%	mg	%
Acide succinique	2.84	4	0	0	0	0	0	0
Acide lactique	4.25	6	8.13	6.7	1.94	4.54	1.7	2.38
Acide pyruvique	18.97	27	49.9	41	—	—	—	—
Alcool	0	0	0	0	0	0	0	0
Acide acétique	28.96	41	46.2	38.1	27	62	40	56
Acide formique	0	0	0	0	0	0	0	0
Anhydride carbonique	14.1	20	21.8	18	12.46	28.4	21.09	29.5
Carbone retrouvé		98		103.8		94.94		87.88

tour, se décomposerait en donnant deux molécules d'acide acétique suivant la réaction étudiée par SLADE ET WERKMAN<sup>33</sup> sur *Aerobacter indologenes*



ce qui expliquerait l'excès d'acide acétique par rapport à l'anhydride carbonique et l'excès d'acide par rapport à l'alcool qui, suivant l'équation de HARDEN<sup>32</sup>, devrait être en proportion équimoléculaire.

Pour vérifier cette réaction sur *E. coli*, nous avons refait nos expériences en anaérobiose, en prenant comme substrat l'acide succinique.

Le Tableau V montre que l'acide succinique se dégrade faiblement en anaérobiose en donnant l'acide fumarique, un pourcentage assez grand de corps en C<sub>2</sub> et presque pas d'anhydride carbonique, ni d'hydrogène; l'hydrogène provenant de la transformation d'acide succinique en acide fumarique a donc été utilisé probablement suivant la réaction de SLADE ET WERKMAN<sup>33</sup>.

On peut alors penser que l'augmentation de corps en C<sub>2</sub> par rapport aux corps en C<sub>1</sub> observée dans nos premières expériences en anaérobiose, provient de la scission d'acide succinique. Mais il faut noter que les expériences faites avec l'acide succinique comme substrat ne sont pas concluantes, car peu de substrat a disparu et on observe toujours, comme dans le bilan du Tableau V, une forte autolyse. Or, dans ce cas, comme nous l'avons déjà souligné, une grande partie de l'acide acétique pourrait provenir de l'autolyse.

Pour déterminer la quantité d'acide acétique qui provient de la scission du succinate, nous avons répété ces mêmes expériences en ajoutant un inhibiteur comme le

TABLEAU V  
ACTION DE *E. COLI* SUR L'ACIDE SUCCINIQUE EN ANAÉROBIOSE

Concentration de l'acide succinique 0.07 M  
Tous les chiffres sont calculés en carbone

Expérience :	r	
<i>Opacité</i> : initiale	112	
finale	56	
Succinate disparu (mg)	18.5	
Produits formés	mg	%
Acide lactique . . . . .	0	0
Acide pyruvique . . . . .	0	0
Alcool . . . . .	0	0
Acide acétique . . . . .	12.7	69
Acide formique . . . . .	0.16	0.86
Anhydride carbonique . . . . .	0.33	1.73
Acide fumarique . . . . .	7.28	39.4
Carbone retrouvé . . . . .		110.99
Hydrogène en ml pour 100 mg de carbone du succinate disparu		0
Azote résiduel (ml) . . . . .		1.82
Oxygène résiduel (ml) . . . . .		0.18
Autolyse notable		

malonate. Mais ces expériences se sont avérées impossibles, l'autolyse étant beaucoup plus forte, les conditions expérimentales deviennent complètement différentes. Nous avons ajouté aussi un pourcentage faible de glucose au succinate, mais sans résultats concluants; néanmoins, les expériences avec succinate donnent une indication en faveur de la scission d'acide succinique en acide acétique. Nous avons essayé aussi de prendre comme substrat un autre diacide en C<sub>4</sub>, le malate, mais il n'a pas été du tout attaqué par *E. coli* dans nos conditions expérimentales.

Il était donc indispensable de recouper nos résultats par une autre méthode afin de voir l'influence directe de l'anhydride carbonique sur la fermentation. Dans ce but, nous avons absorbé l'anhydride carbonique dans les ballons d'expériences avec la technique décrite au début en présence et en absence d'azoture de sodium. Si notre raisonnement est exact, nous devons trouver l'anhydride carbonique théorique correspondant aux corps en C<sub>2</sub> dont le pourcentage doit diminuer. Les expériences *e* (Tableau I), *m* (Tableau III), *e'* et *m'* (Tableau VI) ont été faites avec une même suspension bactérienne dans des conditions expérimentales identiques. Dans le Tableau VII, on a reproduit les moyennes de 7 expériences (le substrat utilisé était toujours le glucose).

On voit immédiatement que le métabolisme est différent en présence ou en l'absence d'anhydride carbonique (ceci attire l'attention sur les erreurs qu'on peut faire pour la détermination des rapports *anhydride carbonique*/*hydrogène* dans l'appareil de Warburg en calculant l'anhydride carbonique, comme il est souvent admis, par la méthode directe). Les différences s'expriment surtout par l'augmentation de l'anhydride carbonique et la diminution d'acide lactique quand on absorbe l'anhydride carbonique. Pour

TABLEAU VI

ACTION DE *E. COLI* SUR LE GLUCOSE EN ANAÉROBIOSE EN ABSENCE D'ANHYDRIDE CARBONIQUE, AVEC  
OU SANS AZOTURE DE SODIUM

Concentration en glucose : 0.02 M

Concentration en azoture : 0.003 M

CO<sub>2</sub> absorbé au fur et à mesure de sa production

Expérience :	e'		m'	
<i>Opacité</i> : initiale	100		100	
finale	80		95	
Glucose disparu (mg)	67.4		62.6	
Produits formés	mg	%	mg	%
Acide succinique . . . . .	5.5	8.16	0	0
Acide lactique . . . . .	8.07	12	28.8	46
Acide pyruvique . . . . .	2.39	3.56	3.2	5.1
Alcool . . . . .	15.85	23.5	7.79	12.4
Acide acétique . . . . .	18.78	27.8	18.7	29.9
Acide formique . . . . .	traces	} 21	traces	} 9.15
Anhydride carbonique . . . . .	14.2		5.74	
Carbone retrouvé . . . . .	—	96.02		102.55
Hydrogène en ml pour 100 mg de carbone du glucose disparu		non dosé		non dosé

les bactéries sans inhibiteur, diminution de 51% d'acide lactique, quantité d'anhydride carbonique environ doublée; pour les bactéries en présence d'azoture, diminution de 15% d'acide lactique, quantité d'anhydride carbonique environ quadruplée. Quand on absorbe l'anhydride carbonique, on observe dans le cas des bactéries sans inhibiteur une légère diminution de corps en C<sub>2</sub>; en présence d'azoture, la proportion des corps en C<sub>2</sub> reste la même avec ou sans absorption du gaz carbonique.

Ainsi, nous voyons d'après les résultats des Tableaux VI et VII qu'avec les bactéries sans inhibiteur, lorsqu'on absorbe le gaz carbonique nous retrouvons les pourcentages théoriques d'anhydride carbonique correspondant aux corps en C<sub>2</sub>, mais, contrairement à ce que l'on attendait, ceci est dû, non pas à une diminution de corps en C<sub>2</sub>, mais à une grande augmentation de l'anhydride carbonique.

Or, si seule était en cause la synthèse d'acide succinique\* à partir d'un corps en C<sub>3</sub> par fixation d'anhydride carbonique et ensuite décomposition en deux corps en C<sub>2</sub>, on devrait observer, dans les expériences avec absorption, une augmentation d'acide lactique et d'anhydride carbonique correspondante et parallèlement une forte diminution de corps en C<sub>2</sub>.

Il faut donc admettre qu'à côté de la réaction de WERKMAN, il y a un autre mode d'utilisation de l'anhydride carbonique par sa fixation sur un corps en C<sub>2</sub> avec formation de corps en C<sub>3</sub>, l'absorption de l'anhydride carbonique empêchant les deux catégories de synthèses. En outre, la quantité d'anhydride carbonique utilisée pour former une partie de l'acide lactique doit être plus grande que celle qui aboutit à la formation d'acide

\* Les pourcentages d'acide succinique sont environ les mêmes avec ou sans absorption d'anhydride carbonique, donc, une partie de l'acide succinique, comme nous l'avons déjà dit, doit provenir, suivant la réaction de SLADE ET WERKMAN, de la condensation de deux molécules d'acide acétique.

TABLEAU VII

ACTION DE *E. COLI* SUR LE GLUCOSE EN ANAÉROBIOSE, EN PRÉSENCE OU EN ABSENCE D'ANHYDRIDE CARBONIQUE, AVEC OU SANS AZOTURE DE SODIUM. MOYENNE DE 7 EXPÉRIENCES

Concentration en glucose: 0.02 M

Concentration en azoture: 0.003 M

Tous les chiffres sont calculés en % de carbone. pH final 6.8

Produits formés calculés pour 100 mg de carbone du glucose disparu	Sans azoture		Avec azoture	
	CO <sub>2</sub> non absorbé	CO <sub>2</sub> absorbé	CO <sub>2</sub> non absorbé	CO <sub>2</sub> absorbé
Ac. succinique	4.9	5.5	traces	traces
Acide lactique	22.63	11	61	51.8
Acide pyruvique	1.71	1.37	4.59	5.27
Alcools	19.85	18.4	9.8	12.1
Acide acétique	36.1	33	27.2	28.1
Acide formique	1.97	0.	1.36	2.07
Anhydride carbonique	10.82	26.8	1.51	8.57
			2.87	10.64
Carbone retrouvé	97.98	96.07	105.46	107.91
Hydrogène (ml)	22	non dosé	1	non dosé

succinique. Dans ce cas, en absorbant le gaz carbonique, on doit diminuer la quantité d'acide lactique, ce qui est en accord avec nos expériences, tandis que les corps en C<sub>2</sub> ne doivent diminuer que légèrement. En effet, la diminution des corps en C<sub>2</sub>, qui aurait dû se produire par suite de la réaction de SLADE ET WERKMAN, se trouve compensée par la non-formation de corps en C<sub>3</sub> à partir de corps en C<sub>2</sub>. Nous voyons que l'anhydride carbonique mesuré au cours d'une fermentation normale du glucose ne correspond qu'à 40% environ de l'anhydride carbonique total dégagé par les bactéries, et trouvé par absorption. Les 60% d'anhydride carbonique restant sont utilisés pour les deux sortes de synthèses que nous avons indiquées: formation de corps en C<sub>3</sub> et de corps en C<sub>4</sub>.

En présence d'azoture de sodium, quand on absorbe le gaz carbonique, on observe, comme nous l'avons déjà noté, une diminution d'acide lactique, le pourcentage des corps en C<sub>2</sub> restant environ le même. Il y a également une très forte augmentation de l'anhydride carbonique par rapport au témoin avec azoture et sans absorption. Mais on ne retrouve pas, cependant, la quantité d'anhydride carbonique correspondant aux corps en C<sub>2</sub>. C'est peut-être une question de vitesse de réaction. En présence d'azoture, les synthèses se feraient plus vite que l'anhydride carbonique ne serait absorbé et il est impossible d'empêcher la totalité des synthèses. Nous avons vu, en effet, dans nos expériences initiales, qu'en présence d'azoture, les synthèses par fixation d'anhydride carbonique sont plus grandes, puisqu'on ne retrouve que 15% de l'anhydride carbonique théorique. Il faut également noter qu'en présence d'azoture, nous obtenons en général des bilans supérieurs à 100, quoique l'autolyse soit toujours très faible; peut-être l'azoture permet-il l'utilisation d'une source de carbone contenue dans les bactéries elles-mêmes. Ceci reste à étudier.

Nous avons essayé de démontrer directement la possibilité de la fixation d'anhydride carbonique sur les corps en C<sub>2</sub> en mettant les bactéries en présence d'acétate ou d'alcool et d'anhydride carbonique. UTTER, LIPMANN ET WERKMAN<sup>34</sup> et LIPMANN ET TUTTLE<sup>35</sup> avaient pu mettre en évidence la formation d'acide pyruvique à partir



TABLEAU VIII

ACTION DE *E. COLI* SUR L'ACÉTATE, EN ANAÉROBIOSE, EN PRÉSENCE OU EN ABSENCE D'ANHYDRIDE CARBONIQUE, AVEC OU SANS AZOTURE DE SODIUM. MOYENNE DE CINQ EXPÉRIENCES

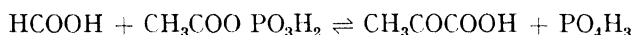
Concentration du glucose : 0.02 M

Concentration de l'azoture de sodium : 0.003 M

PH = 6.9

	Sans CO <sub>2</sub>				Avec CO <sub>2</sub> (atmosphère de N <sub>2</sub> + 5 % CO <sub>2</sub> solution de bicarbonate 0.005 M)			
	Sans azoture		Avec azoture		Sans azoture		Avec azoture	
Opacité: initiale	137		130		137		130	
finale	103		105		115		112	
Acétate disparu (mg)	15.8		7.7		15.8		7.7	
	mg	%	mg	%	mg	%	mg	%
Somme des corps en C <sub>3</sub> formés (acide lactique + acide pyruvique)	0	0	0	0	0.63	4	0.7	9.2

du formiate et de l'acétylphosphate par un extrait de *E. coli* à l'aide de l'isotope C<sup>13</sup> suivant la réaction réversible :



L'équilibre de cette réaction étant dans le sens acétylphosphate et formiate, il se forme des quantités extrêmement faibles d'acide pyruvique<sup>36</sup>.

Nous avons fait nos expériences sur des bactéries intactes en les mettant en suspension, soit dans un tampon phosphate dans les conditions de nos expériences précédentes, soit dans un tampon de phosphate auquel on ajoute du bicarbonate (concentration finale M/20), dans une atmosphère d'azote contenant 5 % de gaz carbonique (Tableau VIII).

Puisque les bactéries sans substrat produisent seulement des traces de corps en C<sub>3</sub>, on pouvait se permettre de faire dans ces expériences des témoins dans les mêmes conditions expérimentales, c'est-à-dire mettant en jeu des bactéries sans substrat avec ou sans anhydride carbonique, laissées 22 heures à l'étuve à 37°. Les chiffres très faibles obtenus dans ces conditions sont retranchés des résultats du Tableau VIII.

Nous voyons, d'après ces résultats, qu'il ne se forme aucun corps en C<sub>3</sub> (somme acide lactique + acide pyruvique) avec les bactéries en présence d'acétate sans anhydride carbonique, tandis que lorsqu'on ajoute de l'anhydride carbonique, il y a un pourcentage notable de corps en C<sub>3</sub>. Nous avons essayé de remplacer le bicarbonate par le formiate, mais les quantités de corps en C<sub>3</sub> obtenues étaient difficilement dosables. Nous avons répété ces mêmes expériences en présence d'azoture de sodium (Tableau VIII). Nous retrouvons les résultats précédents, c'est-à-dire que l'azoture n'empêche pas la synthèse des corps en C<sub>3</sub> par fixation d'anhydride carbonique sur les corps en C<sub>2</sub>.

Nous considérons que les pourcentages obtenus de corps en C<sub>3</sub> en présence ou en l'absence d'azoture sont relativement élevés. Il ne faut pas oublier, en effet, que nous travaillons avec des bactéries intactes et que les substrats ajoutés de l'extérieur ne sont pas utilisés avec la même intensité que lorsqu'ils sont formés au cours du métabolisme.

TABLEAU IX

ACTION DE *E. COLI* SUR L'ALCOOL ÉTHYLIQUE ET SUR LE PHOSPHATE D'ÉTHYLE EN ANAÉROBIOSE, EN PRÉSENCE OU EN ABSENCE D'ANHYDRIDE CARBONIQUE

Concentration en alcool ou en phosphate d'éthyle : 0.017 M

pH = 6.9

Les expériences avec l'anhydride carbonique sont faites en présence de bicarbonate de sodium 0.05 M, en atmosphère N<sub>2</sub> + 5 % CO<sub>2</sub>

	Expérience I				Expérience II			
	Alcool		Phosphate d'éthyle		Alcool		Phosphate d'éthyle	
	Sans CO <sub>2</sub>	Avec CO <sub>2</sub>	Sans CO <sub>2</sub>	Avec CO <sub>2</sub>	Sans CO <sub>2</sub>	Avec CO <sub>2</sub>	Sans CO <sub>2</sub>	Avec CO <sub>2</sub>
Opacité : initiale	146	146	146	146	161	161	161	161
finale	121	132	118	127	112	121	108	118
Produits formés (mg)								
Acide lactique + acide pyruvique	0	1.76	0.43	4.02	0	0.3	0.65	1.87

Avec l'alcool comme substrat, les résultats obtenus ne sont pas constants, du fait que l'alcool n'est pas toujours attaqué par *E. coli* dans nos conditions expérimentales. On obtient pourtant, dans quelques expériences, une formation de corps en C<sub>3</sub> en présence d'anhydride carbonique. Peut-être y a-t-il une question de phosphorylation, car le phénomène est beaucoup plus net lorsqu'on remplace l'alcool éthylique par le phosphate d'éthyle\* (Tableau IX). Il faut noter, d'ailleurs, qu'avec le phosphate d'éthyle, il se synthétise, en plus, probablement, d'autres corps. En effet, dans ce cas, lorsqu'on fait le dosage d'acide pyruvique par la carboxylase, on observe un dégagement d'anhydride carbonique, alors même que le dosage d'acide pyruvique est terminé. Suivant KREBS<sup>27</sup>, ceci est dû à la présence d'autres corps comme l'acide oxalactique ou l'acide α-cétoglutarique.

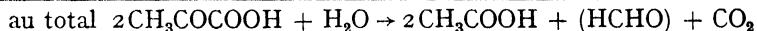
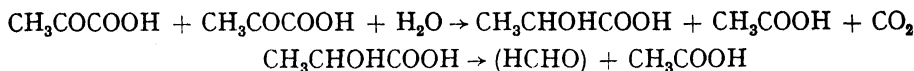
## DISCUSSION

Depuis WURMSER<sup>38</sup>, on distingue les fermentations liées aux synthèses, des fermentations indépendantes. Et la justification de cette conception a été apportée, entre autres, par FROMAGEOT ET SAFAVI<sup>12</sup> et par CLIFTON<sup>2, 3</sup> et CLIFTON ET LOGAN<sup>4, 6</sup>. Mais les produits synthétisés peuvent avoir deux devenir : comme CLIFTON l'a fait remarquer, ils peuvent être assimilés, ce qui se traduit par une augmentation du poids des matières vivantes, ou rester dans le milieu de culture. D'après les auteurs dont nous venons de parler, le produit assimilé répond à la formule (HCHO)<sub>n</sub>. Il provient, soit de l'oxydation incomplète dans les expériences de CLIFTON, de l'acide butyrique ou de l'acide acétique, par exemple :



\* Le phosphate d'éthyle était préparé dans notre laboratoire, selon la méthode de O. BAILLY<sup>37</sup> par R. SUTRA, que nous sommes heureux de remercier ici.

soit de la dégradation fermentaire incomplète de l'acide lactique dans les expériences de FROMAGEOT ET SAFAVI:



On pourrait interpréter de façon analogue les résultats de WINZLER ET BAUMBERGER<sup>10</sup> et de VAN NIEL ET ANDERSON<sup>11</sup>. Tout cela est hors de doute et parfaitement cohérent. Mais dans ces expériences, il y avait formation de matière vivante. Lorsque les auteurs travaillaient avec des bactéries non proliférantes, il n'était plus question de ce corps  $(\text{HCHO})_n$  qui devait être assimilé.

Or, dans nos expériences, faites précisément avec des bactéries à l'état non proliférant, si les bilans faits en aérobiose montrent qu'aucune synthèse n'a été constatée, il n'en est pas de même en anaérobiose et précisément le corps synthétisé, mais non assimilé dans notre cas, est l'acide lactique  $(\text{HCHO})_3$ , du moins pour la part la plus importante, car, on le verra, il y a aussi de l'acide succinique et de l'acide pyruvique synthétisés.

Cet acide lactique provient, comme l'acide pyruvique, d'ailleurs, d'une fixation d'anhydride carbonique sur les corps en  $\text{C}_2$ . L'origine de ces deux acides est donc double: la dégradation du glucose selon le schéma classique, et la synthèse. Quant à l'acide succinique, comme WERKMAN l'a déjà montré, il provient pour une part de la condensation de l'acide acétique suivant la réaction de THÜNBERG<sup>39</sup>, et, pour une autre part, de la fixation de l'anhydride carbonique sur les acides en  $\text{C}_3$ .

Il est possible, d'après nos résultats expérimentaux (Tableaux VI et VII), de calculer approximativement les quantités d'anhydride carbonique utilisées pour chaque synthèse.

Appelons  $x$  le pourcentage de carbone du glucose qui, à l'état d'anhydride carbonique se fixe sur les corps en  $\text{C}_2$  et  $y$  celui qui se fixe sur les corps en  $\text{C}_3$ .

On constate (Tableau VII) que, dans le cas où l'anhydride carbonique est absorbé, donc ne peut pas réagir, il y a, par rapport aux expériences sans absorption, une augmentation du pourcentage en carbone des corps en  $\text{C}_1$  de  $26.8 - 12.79 = 14$ . Nous pouvons donc écrire:

$$x + y = 14$$

Mais lorsqu'on absorbe l'anhydride carbonique, les corps en  $\text{C}_2$  augmentent par suite de la non-fixation de l'anhydride carbonique pour les transformer en corps en  $\text{C}_3$ . Ceci représente un pourcentage en carbone de  $2x$ . Mais nous avons vu que l'acide succinique est, lui aussi, générateur de corps en  $\text{C}_2$ . La non-fixation de l'anhydride carbonique sur les corps en  $\text{C}_3$  diminue de  $4y$  le pourcentage en carbone des corps en  $\text{C}_2$  qui se forment à partir de l'acide succinique. Comme l'expérience montre que, dans le cas de non-emploi de l'anhydride carbonique, on a une différence pour les corps en  $\text{C}_2$  de  $51.4 - 55.95 = -4.5$ , nous pouvons écrire:

$$2x - 4y = -4.5$$

Des deux équations on tire:

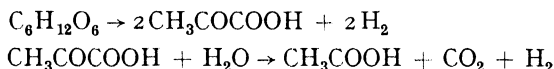
$$x = 8.6 \quad y = 5.4$$

A l'aide de ces deux valeurs, on peut calculer la diminution d'acide lactique qui doit exister quand on absorbe l'anhydride carbonique, et comparer cette valeur avec celle trouvée expérimentalement. Les deux résultats sont concordants.

Comme on a au total 14% de carbone de l'anhydride carbonique utilisé pour les synthèses, et comme la valeur de  $x$  est 8.6 et celle de  $y$  est 5.4, on peut calculer les pourcentages respectifs d'anhydride carbonique utilisé pour chaque sorte de synthèse. Pour la synthèse des corps en  $C_4$ , il y en a  $5.4 : 14 = 38\%$  environ, et pour la synthèse des corps en  $C_3$ , 62% environ.

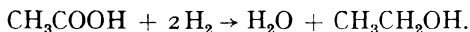
Voici une vérification : Dans les expériences e et e' (Tableaux I et VI), on voit qu'il y a 7% (21 — 14.28) environ de carbone de l'anhydride carbonique utilisé pour les synthèses. Pour la synthèse des corps en  $C_3$ , le pourcentage de carbone de l'anhydride carbonique utilisé sera de :  $7 (62 : 100) = 4.3$ . Celui utilisé pour la synthèse des corps en  $C_4$  :  $7 (38 : 100) = 2.7$ . On devrait donc avoir, dans le cas de l'absorption de l'anhydride carbonique, une diminution des corps en  $C_3$  correspondant à :  $3(4.3 - 2.7) = 5\%$  environ, et pour les corps en  $C_2$  une diminution de :  $(2 \cdot 4.3) - (4 \cdot 2.7) = 2.2\%$  environ. Or, dans l'expérience, nous observons pour les corps en  $C_3$  une diminution de 6% et pour les corps en  $C_2$  une diminution de 2%.

La quantité d'hydrogène trouvée dans nos expériences ne correspond pas, elle non plus, à la quantité théorique qu'on peut calculer suivant les réactions de la fermentation classique :

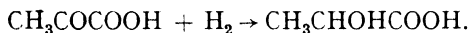


Il devrait y avoir, pour 100 mg de carbone du glucose disparu (Tableau VII), 114 mg d'hydrogène. Or, on ne retrouve que :

37 ml d'hydrogène utilisé pour former l'alcool suivant la réaction :



14 ml d'hydrogène utilisé pour former l'acide lactique suivant la réaction :

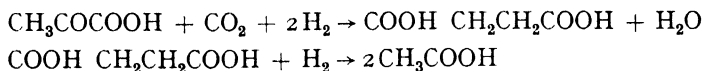


3.6 ml d'hydrogène restant sous forme d'acide formique.

22 ml d'hydrogène dégagé pendant la fermentation.

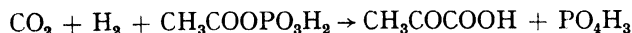
Donc, au total, 76.6 ml, ce qui correspond seulement à 67% de la quantité de l'hydrogène théorique.

Quand on admet les calculs précédents pour les deux synthèses que nous avons mises en évidence, on peut expliquer l'utilisation de l'hydrogène manquant dans le bilan. En effet, la formation d'acide acétique par le schéma de WOOD ET WERKMAN, et de SLADE ET WERKMAN exige de l'hydrogène :



Le pourcentage du carbone provenant de l'acide acétique formé par cette voie est de  $5.4 \cdot 4 = 21.6$ , puisque le pourcentage de carbone de l'anhydride carbonique fixé sur les corps en  $C_3$  est de 5.4 (voir les calculs précédents). L'hydrogène utilisé dans cette réaction est de :  $(67.2 \cdot 25.6) : 48 = 32$  ml.

La formation d'acide pyruvique par fixation d'anhydride carbonique sur les corps en  $C_2$  exige également de l'hydrogène :



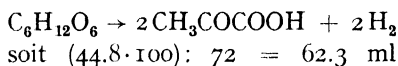
Le pourcentage du carbone de l'anhydride carbonique fixé sur les corps en  $C_2$  étant de

8.6, le pourcentage du carbone de l'acide pyruvique formé selon ce schéma est de:  $8.6 \cdot 3 = 25.8$ . L'hydrogène utilisé est de:  $(22.4 \cdot 25.8) : 36 = 16$  ml.

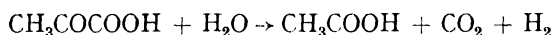
On retrouve donc au total 124 ml d'hydrogène utilisé.

D'autre part, la quantité d'hydrogène produite par fermentation quand on admet les calculs cités plus haut pour les deux synthèses, est égale à:

1. celle dégagée lors de la formation d'acide pyruvique

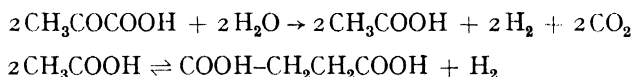


2. celle dégagée lors de la formation des corps en  $\text{C}_2$  par le schéma classique:



Le pourcentage du carbone provenant de la quantité totale de corps en  $\text{C}_2$  formé est de:  $55.95 + 17.2 = 73.12$  (17.2 étant le pourcentage du carbone des corps en  $\text{C}_2$  formés qui ont été utilisés pour la synthèse des corps en  $\text{C}_3$ , soit  $8.6 \cdot 2$ ). Comme 21.6% du carbone provient des corps en  $\text{C}_2$  formés par la réaction de SLADE ET WERKMAN, on a:  $73.12 - 21.6 = 51.52\%$  du carbone provenant des corps en  $\text{C}_2$  formés par le schéma classique. La quantité d'hydrogène produit est donc:  $(22.4 \cdot 51.52) : 24 = 48.5$  ml.

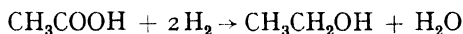
3. la quantité d'hydrogène dégagé lors de la formation d'acide succinique par la réaction réversible de SLADE ET WERKMAN



qui est égale à:  $(67.2 \cdot 4.9) : 48 = 6.8$  ml.

La quantité d'hydrogène totale produite par ces trois réactions est de 118 ml, donc en accord avec celle trouvée dans l'expérience: 124 ml.

On sera peut-être surpris de voir que nous considérons que l'alcool est produit par la réaction:



Mais il y a de bonnes raisons pour qu'il en soit ainsi. L'origine de l'alcool par décarboxylation de l'acide pyruvique en acétaldéhyde et réduction de l'acétaldéhyde ne se produit pas avec *E. coli*. VIRTANEN ET TIKKA<sup>40, 41</sup> l'ont montré. D'autre part, OSBURN, BROWN ET WERKMAN<sup>42</sup>, MICKELSON ET WERKMAN<sup>43</sup> et PEYNAUD<sup>44</sup> ont montré la réduction bactérienne d'acides en alcool, et en particulier, SLADE ET WERKMAN<sup>33</sup> ajoutant dans un milieu où le glucose fermente sous l'action d'*Aerobacter indologenes* de l'acide acétique  $\text{CH}_3\text{C}^{13}\text{OOH}$ , contenant 2.39% de  $\text{C}^{13}$ , retrouvent dans l'alcool éthylique 1.64% de carbone 13. Nous-mêmes avons des expériences indicatives permettant de penser que le processus de formation de l'alcool éthylique par *E. coli* est une réduction de l'acide acétique.

En ce qui concerne nos expériences avec l'azoture de sodium, celui-ci, loin d'inhiber la réaction de fixation de l'anhydride carbonique sur les corps en  $\text{C}_2$  ou en  $\text{C}_3$ , favorise au contraire des réactions synthétiques. Comme cet inhibiteur empêche la croissance de *E. coli*\*, il faut en conclure, ce qui est en accord avec CLIFTON ET LOGAN<sup>4-6</sup> et SPIEGELMAN<sup>18</sup>, que l'azoture inhibe les phénomènes d'assimilation.

\* Nous avons isolé pourtant un mutant de *E. coli*, qui peut se développer en présence d'azoture de sodium à la concentration M/350.

## RÉSUMÉ

L'examen des bilans de produits de fermentation du glucose par *Escherichia coli* à l'état non proliférant, montre, en anaérobiose, un déficit d'anhydride carbonique et d'hydrogène qui s'explique par un réemploi de ces corps pour des synthèses. En particulier, on a montré la fixation de l'anhydride carbonique sur les corps en  $C_2$ , acide acétique, alcool, phosphate d'éthyle, pour donner des acides en  $C_3$ , et sur des acides en  $C_3$  pour donner des diacides en  $C_4$ . Il est possible de calculer la part de l'anhydride carbonique utilisé pour chacune des synthèses. Celles-ci sont favorisées par l'azide.

## SUMMARY

The examination of the distribution of the products of the fermentation of glucose by non-proliferating *Escherichia coli* shows, under anaerobic conditions, a deficit of  $CO_2$  and  $H_2$ . This can be explained by the re-use of these substances for syntheses. In particular, it has been shown that  $CO_2$  combines with  $C_2$ -compounds such as acetic acid, alcohol, ethyl phosphate to give  $C_3$ -acids, and with  $C_3$ -acids to give dibasic  $C_4$ -acids. It is possible to calculate the amount of  $CO_2$  used for each of these syntheses which are favoured by azide.

## ZUSAMMENFASSUNG

Die Bilanz der Produkte der Vergärung von Glucose durch nicht proliferierende *Escherichia coli* in Anaerobiose, ergibt einen Defizit an Kohlendioxyd und Wasserstoff der sich durch erneute Verarbeitung dieser Substanzen in Synthesen erklären lässt. Insbesondere wurde die Bindung von Kohlendioxyd an  $C_2$ -Verbindungen, wie Essigsäure, Alkohol, Äthylphosphat, unter Entstehung von  $C_3$ -Säuren und die Bindung an  $C_3$ -Säuren unter Entstehung von zweibasischen  $C_4$ -Säuren gezeigt. Es ist möglich, die für jede dieser Synthesen verbrauchte Menge Kohlendioxyd zu berechnen. Die Synthesen werden durch Azid gefördert.

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## THE EFFECT OF SULFHYDRYL COMPOUNDS, PENICILLIN, AND COBALT ON THE CELL DIVISION MECHANISM OF YEASTS

by

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The production of elongated cells of microorganisms may be viewed as a result of the differential inhibition of cell division without a concomitant inhibition of metabolic processes necessary for the growth of cells. This concept has been elaborated for bacteria<sup>1, 2</sup> and for yeasts and yeast-like organisms<sup>3, 4, 5, 6</sup>. There is evidence which indicates that differential inhibition of cell division results from the inhibition of a single enzyme complex, specifically active in cell division<sup>3</sup>. The cell division enzyme appears to be sensitive to intracellular sulfhydryl  $\rightleftharpoons$  disulfide equilibria, and to be particularly sensitive to the presence of penicillin and cobalt. As is well known, there is a very extensive literature indicating that sulfhydryl groups ( $-SH$ ) play some special rôle in cell division. RAPKINE<sup>7, 8</sup> has clearly implicated  $-SH$  groups in the cell multiplication processes of yeasts. Early studies on the effect of sub-bacteriostatic concentrations of penicillin by GARDNER<sup>9</sup> demonstrated that elongation of bacterial cells into filaments (designated here as  $B \rightarrow F$  conversions) is the first, microscopically demonstrable, effect of penicillin. It is important to stress the extreme specificity of the locus affected by penicillin at such high dilution. Recently PRATT AND DUFRENOY<sup>10</sup> have emphasized this aspect of penicillin action and have demonstrated both an *in vivo* and an *in vitro* enhancement of penicillin by trace amounts of cobalt. The action of cobalt on yeast to mycelial conversion ( $Y \rightarrow M$ ) was observed independently, as an outcome of studies on the accumulation of radioactive cobalt by yeasts<sup>11</sup>.

The present studies concern the action of sulfhydryl compounds on the inhibition of  $Y \rightarrow M$  (promotion of cell division), and the promotion of  $Y \rightarrow M$  (inhibition of cell division) by penicillin and heavy metals. Preliminary cytological studies on the nuclear conditions accompanying  $Y \rightarrow M$  conversions have also been made. These indicate that nuclear division may continue even though cell division has been inhibited.

### MATERIALS AND METHODS

Several species of yeasts, selected from the cultures maintained at the CENTRAAL BUREAU VOOR SCHIMMELCULTURES, Yeast Division, Delft, were employed as test organisms in a study of the effects of a variety of chemical substances on yeast to mycelial conversions ( $Y \rightarrow M$ ). The following organisms (strain number indicated) were used:

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<i>Candida albicans</i> ,	35.1. 1	<i>Torulopsis colliculosa</i> ,	24.3. 1
<i>Candida pulcherrima</i> ,	35.2. 1	<i>Torulopsis Molischiana</i> ,	24.5. 1
<i>Candida tropicalis</i> ,	35.5. 1	<i>Torulopsis utilis</i> ,	24.9. 1
<i>Hansenula anomala</i> ,	10.1. 1	<i>Trichosporon capitatum</i> ,	46.5. 1
<i>Saccharomyces cerevisiae</i> ,	8.1. 1	<i>Trichosporon sp.</i> ,	46.7. 1

The slide culture technique<sup>12</sup> was employed to permit microscopic examination of Y → M development. Addition of a cover glass to the agar slide cultures facilitated prolonged microscopic inspection. A potato agar was employed as the basal medium to which all substances to be tested were added; this was prepared as follows:

potato extract	230 ml
tap H <sub>2</sub> O	770 ml
glucose	20 g
agar	20 g

The potato extract was prepared by soaking 100 g of washed, peeled, and ground potatoes in 300 ml tap water for several hours at ca. +4° C. The mass was then filtered through cloth and autoclaved for 15 minutes at 1<sup>3</sup>/<sub>4</sub> atmospheres.

All substances to be tested were sterilized separately by heating or by filtration, as appropriate, and added to the potato agar immediately before preparing the slide cultures to give final concentrations as indicated. All slides were inoculated promptly (in duplicate) with three parallel streaks, using 24–48 hours old stock cultures. Microscopic examinations were made after 24 and 48 hours incubation in a humid atmosphere (in sterile Petri dishes) at 25° C. The organic chemicals employed were commercial products, purified when necessary before use; sodium penicillin G was a product of the NEDERLANDSCHE GIST - EN SPIRITUS-FABRIEK; inorganic compounds used were reagent quality.

Cytological studies were made on air dried smears stained for ten minutes with 1% aqueous toluidine blue (National Aniline), washed in water, and differentiated in 95% ethanol.

#### EXPERIMENTAL

The slide culture technique, a modification of the direct agar-microscopy procedure of ØRSKOV<sup>33</sup>, is a quite satisfactory method for determining whether or not a given strain of a yeast will develop mycelial or pseudo-mycelial structures. This method is widely employed for purposes of identification of yeasts. The potato agar substrate, usually employed with the slide culture method, permits ready development of M structures by strains of the *Mycotoruloideae*, which are recognized on the basis of their production of such structures. This procedure can also serve as a suitable method for the testing of substances, added to the potato agar, for their effect on Y → M conversions. In this manner we have tested many compounds for ability to inhibit or to promote Y → M conversions in a variety of yeasts.

##### *Maintenance of a single cell condition (Inhibition of Y → M)*

As a result of work<sup>4, 5</sup> on the fractionation of filtrates (from cultures of *Trichophyton rubrum*) which exhibited ability to inhibit Y → M in *Candida albicans*, our attention was directed toward the testing of sulfhydryl (–SH) group containing compounds for such properties. As shown in Table I the property of inhibiting Y → M is clearly exhibited by cysteine at 10<sup>–2</sup> M concentration in potato agar and is apparent, with *C. albicans* and *H. anomala*, at 10<sup>–3</sup> M. The appearance of cysteine-treated, and of control growths, is shown in Figs 1, 2, and 6a. A simple influence on the redox potential as an explanation for the action of cysteine appears improbable from the data in Table II which show a complete absence of effect with ascorbic acid at 10<sup>–2</sup> M (Fig. 4a and b). A certain specificity in the nature of the –SH donor may also be inferred from Table III, wherein it is shown that sodium thioglycollate (Fig. 3) is less effective than glutathione, which is in turn less effective than cysteine for inhibiting Y → M. The high

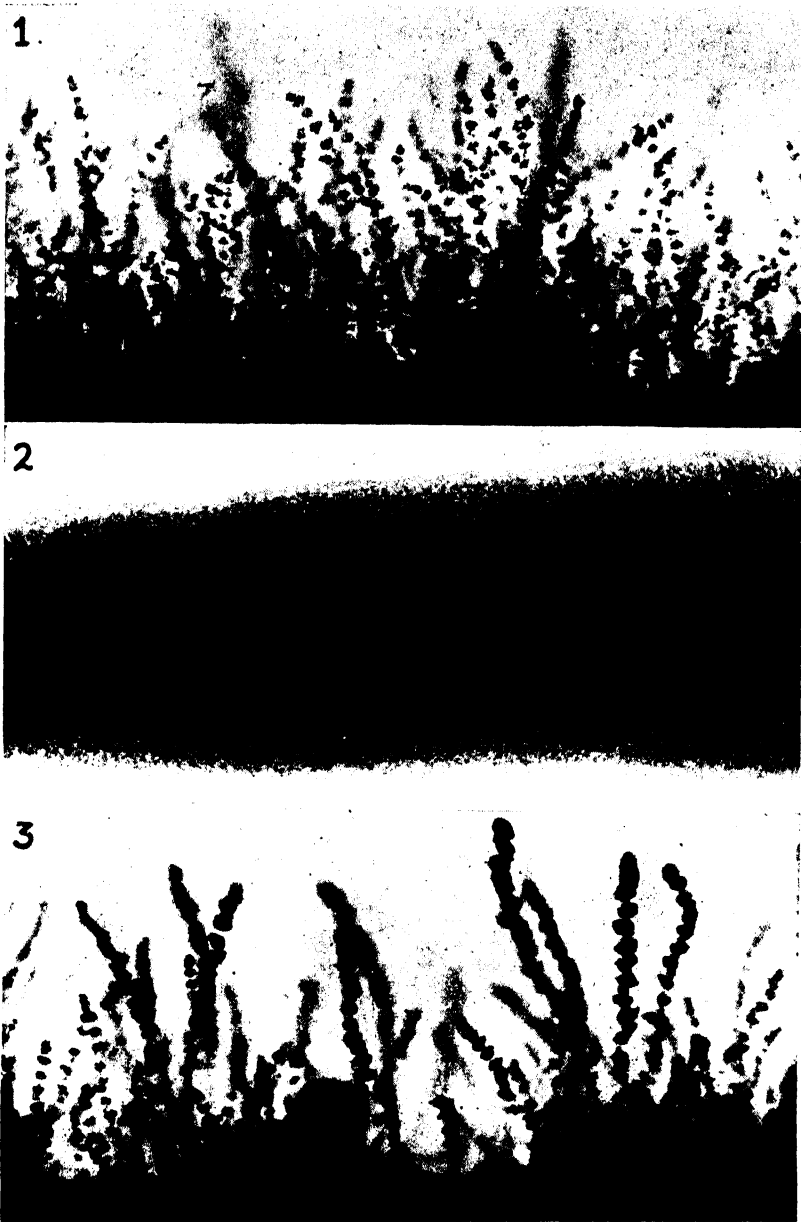


Fig. 1. *Candida albicans*, untreated, from control slide culture; note spacing between clusters of blastospores; 100  $\times$

Fig. 2. *Candida albicans*, slide culture treated with M/100 cysteine; complete inhibition of M development; 100  $\times$

Fig. 3. *Candida albicans*, slide culture treated with M/100 sodium thioglycollate; note close spacing of clusters of blastospores; 100  $\times$

activity of potassium dithiooxalate is notable. While this compound exhibited considerable growth-inhibitory action when tested against yeasts that do not normally form M structures (*Torulopsis Molischiana*, *T. colliculosa*, and *Saccharomyces cerevisiae*), it adversely affected the growth of only one of the yeasts listed in Table III.

Inhibition of mycelial formation may also be viewed as the maintenance of cells in a single cell condition. In this light we may also consider the action of cysteine to be one of favouring, or promoting cell division. This concept of association of -SH compounds with cell division may serve to clarify many earlier observations on the dimorphism of *Candida albicans* in which various metabolic phenomena were implicated. As is well known (see HEWITT<sup>13</sup> and KLUYVER<sup>14</sup>), the metabolism of carbohydrate substrates by yeasts is associated with a fall in the redox potential of the medium (reflecting, in all probability to varying degrees, conditions in the intracellular environment). This intracellular reducing situation will undoubtedly shift the *in vivo* equilibrium,  $-SH \rightleftharpoons -S-S-$ , toward the left. Early researches of LIROSSIER AND ROUX<sup>15</sup>, and FINEMAN<sup>16</sup>, on  $Y \rightarrow M$

TABLE I

EFFECT OF CYSTEINE ON MAINTENANCE OF SINGLE CELL CONDITION AND INHIBITION OF  $Y \rightarrow M$  CONVERSIONS IN DIFFERENT YEASTS

— = NONE OBSERVABLE; +, ++, AND +++ INDICATE SLIGHT, MODERATE, AND PRONOUNCED, RESPECTIVELY

Organism	Control (Potato agar)		Cysteine ( $10^{-3}$ M)		Cysteine ( $10^{-2}$ M)	
	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth
<i>C. albicans</i> . . . . .	+++	+++	++	+++	—	++++
<i>C. tropicalis</i> . . . . .	+++	+++	+++	+++	—	+++
<i>Trich. capitatum</i> . . .	+++	+++	+++	+++	++	+++
<i>H. anomala</i> . . . . .	+	+++	—	+++	—	+++

TABLE II

EFFECT OF ASCORBIC ACID ON  $Y \rightarrow M$  CONVERSIONS

Organism	Control		Ascorbic acid ( $10^{-3}$ M)		Ascorbic acid ( $10^{-2}$ M)	
	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth
<i>C. albicans</i> . . . . .	+++	+++	+++	+++	+++	+++
<i>C. tropicalis</i> . . . . .	+++	+++	+++	+++	++++	+++
<i>Trichosporon sp.</i> . . .	+++	+++	+++	+++	+++	+++
<i>H. anomala</i> . . . . .	++	+++	+++	+++	++	+++

TABLE

EFFECT OF DIFFERENT —

Organism	Control		Sodium thioglycollate ( $10^{-2}$ M)		Sodium thioglycollate ( $10^{-3}$ M)	
	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth
<i>C. albicans</i> . .	+++	+++	+++	+++	+	+++
<i>C. tropicalis</i> . .	+++	+++	+++	+++	+++	+++
<i>Trich. capitatum</i>	+++	+++	+++	+++	+++	+++
<i>H. anomala</i> . .	++	+++	+++	+++	++	+++

conversions in *C. albicans*, can be seen to indicate that readily metabolizable carbon sources (e.g., glucose) inhibit  $Y \rightarrow M$ , while carbon substrates metabolized slowly, or hardly at all, favour  $Y \rightarrow M$ . It is clear that in the latter case the sulfhydryl  $\rightleftharpoons$  disulfide equilibrium will lie towards the right. There are also the well known observations of LANGERON AND GUERRA<sup>17</sup> on the failure of M development on the inner sides of *closely-spaced* parallel streaks of *C. albicans*, with abundant  $Y \rightarrow M$  conversions developed on the outer sides of the same streaks. Diffusion of  $Y \rightarrow M$  inhibitors between adjacent streaks was advanced as an explanation of the observed phenomenon<sup>17</sup>. Recently, MAGNI<sup>18</sup> has confirmed these observations; he found, furthermore, that removal of the agar from one side of an isolated streak (leaving up to 8 mm of agar adjacent to the streak) prevented the appearance of M on the side with a narrow agar boundary, but did not interfere with abundant M production on the opposite side of the streak. The observations of LANGERON AND GUERRA and of MAGNI would seem to be readily accounted for in terms of the metabolic production of a higher concentration of diffusible reducing substance on one side of a streak (sufficiently high on one side to inhibit  $Y \rightarrow M$ ).

#### THE UNCOUPLING OF CELL DIVISION FROM GROWTH

##### Promotion of $Y \rightarrow M$

As already pointed out<sup>9</sup> one of the very first effects of penicillin on bacteria that is observable is the selective inhibition of cell division, without the simultaneous inhibition of other metabolic processes resulting, in many instances, in the growth of bacteria into elongated filaments. From Tables IV and V it will be seen that  $Y \rightarrow M$  was promoted by a concentration of penicillin which was without visible effect on the amount of growth of the test organisms used under our conditions. Addition of cysteine completely antagonized the penicillin effect (we have not yet investigated the minimum concen-

TABLE IV  
EFFECT OF PENICILLIN ON THE UNCOUPLING OF CELL DIVISION FROM GROWTH OF YEASTS, AND THE ANTAGONISM OF PENICILLIN BY CYSTEINE

Organism	Penicillin (11 U/ml)		Penicillin + Cysteine ( $10^{-2}$ M)		Control	
	$Y \rightarrow M$	Growth	$Y \rightarrow M$	Growth	$Y \rightarrow M$	Growth
<i>C. albicans</i> . .	++++	+++	—/+	+++	+++	+++
<i>C. tropicalis</i> . .	++++	+++	—/+	+++	+++	+++
<i>H. anomala</i> . .	+++	+++	—	+++	++	+++

II

##### COMPOUNDS ON $Y \rightarrow M$ CONVERSIONS

Potassium dithiooxalate ( $10^{-3}$ M)		Glutathione ( $3.6 \cdot 10^{-3}$ M)		Cysteine ( $10^{-2}$ M)	
$Y \rightarrow M$	Growth	$Y \rightarrow M$	Growth	$Y \rightarrow M$	Growth
++	+++	+	++++	—	+++
+	+++	++	++++	—	++++
+	+	+++	+++	—/+	+++
—	+++	++	+++	—	+++

References p. 474/475.

TABLE V  
EFFECT OF PENICILLIN AND OF CYSTEINE ON B → F IN *Bacillus cereus*

Penicillin (11 U/m)		Penicillin + Cysteine ( $10^{-2}$ M)		Control	
B → F	Growth	B → F	Growth	B → F	Growth
++++	++	—	+++	+	+++

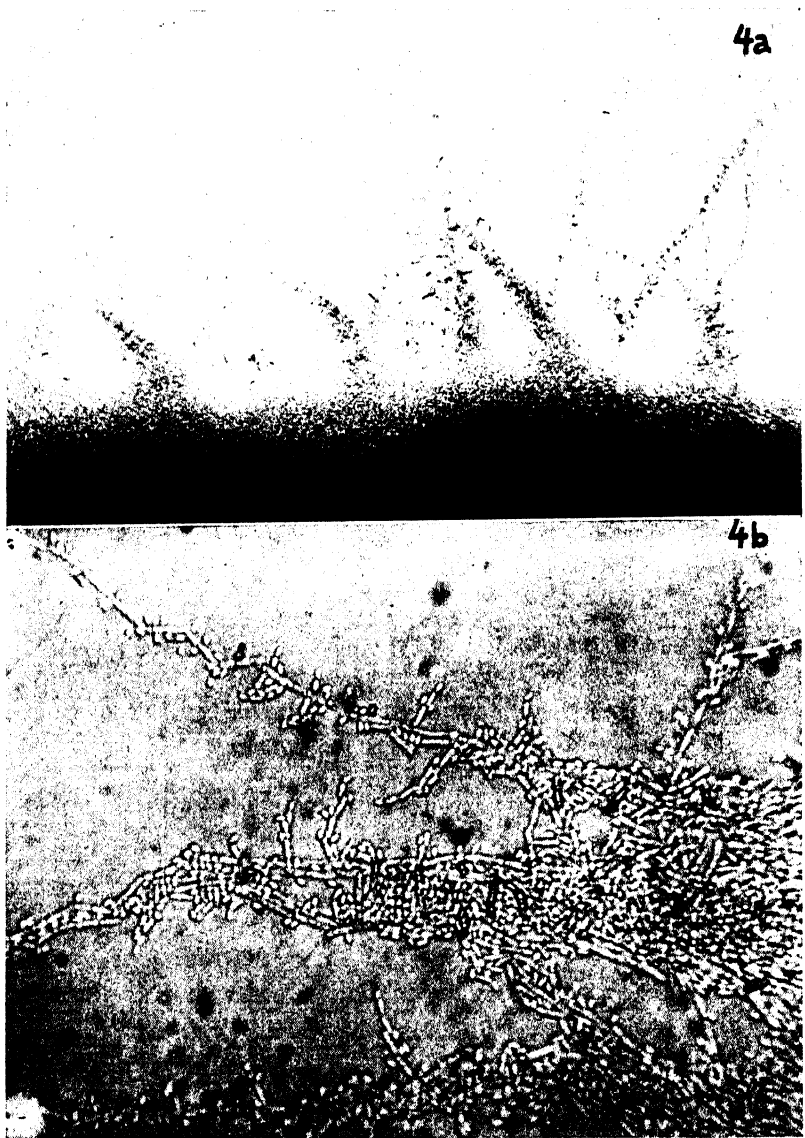


Fig. 4. a) *Hansenula anomala*, from slide culture treated with M/100 ascorbate; filamentous tendencies pronounced; 100 × ; b) same, 400 ×

References p. 474/475.

tration of cysteine necessary for penicillin antagonism under these conditions). With *Bacillus cereus* (Delft strain E XI.1.11.4) as test organism there was a most remarkable increase in cell length with penicillin treatment; while cysteine completely suppressed  $B \rightarrow F$  tendencies. The *B. cereus* slide cultures with cysteine contained a very high percentage of sporulating cells.

Although we have not observed mycelial production with cells of *Saccharomyces cerevisiae* exposed to penicillin (or to other treatment), a definite change in cell morphology has been regularly observed. As shown in Fig. 5a the cell shape in penicillin treated slide cultures is considerably more elongate than that of the normal-appearing cells from penicillin plus cysteine treatment (Fig. 5b).

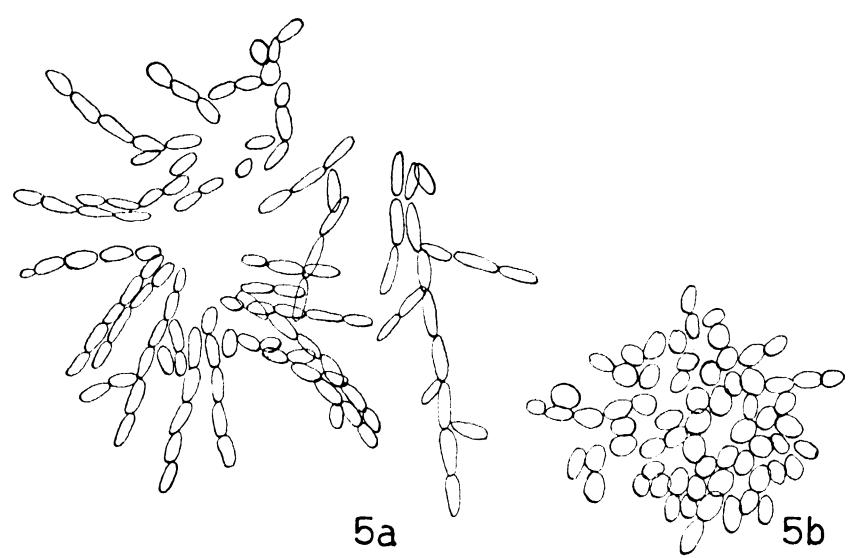


Fig. 5. a) *Saccharomyces cerevisiae*, camera lucida drawing of cells from slide agar culture treated with penicillin (see text); cells elongated, 440  $\times$ ; b) same, from agar culture with penicillin plus M/100 cysteine, 440  $\times$

In some earlier investigations, in which -SH groups were associated with cell division of microorganisms, emphasis was placed on the inhibitory action of heavy metals on cell division processes. The  $Cu^{++}$  ion was found by VOEGTLIN AND CHALKLEY<sup>19</sup> to be especially toxic for the cell division of *Ameba proteus*. Cobalt enhancement of the action of penicillin both *in vitro* and *in vivo*<sup>10</sup> has already been mentioned. We have studied the effect of certain heavy metals on  $Y \rightarrow M$  processes in several yeasts. Table VI

TABLE VI  
EFFECT OF COBALT ON THE PROMOTION OF  $Y \rightarrow M$

Organism	Control		CoAc <sub>2</sub> ( $4 \cdot 10^{-5}$ M)		CoAc <sub>2</sub> ( $10^{-3}$ M)	
	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth	Y $\rightarrow$ M	Growth
<i>C. albicans</i> . . . . .	+++	+++	+++	+++	+++++	+++
<i>C. tropicalis</i> . . . . .	+++	+++	++++	+++	+++++	+++
<i>Trich. capitatum</i> . . . .	+++	+++	+++	+++	+++++	+++
<i>H. anomala</i> . . . . .	++	+++	+++	+++	+++	+++

References p. 474/475.

T<sub>2</sub>

## EFFECT OF COBALT AND OTHER HEAVY METALS ON Y → M

Organism	Control		CuSO <sub>4</sub> (10 <sup>-3</sup> M)		K <sub>2</sub> B <sub>4</sub> O <sub>7</sub> (10 <sup>-3</sup> M)	
	Y → M	Growth	Y → M	Growth	Y → M	Growth
<i>C. pulcherrima</i>	—	+++	—	+++	—	+++
<i>T. colliculosa</i>	—	+++	—	+++	—	+++
<i>T. Molischiana</i>	—	+++	—	+++	+/-	+++
<i>T. utilis</i>	—	+++	+	+++	+	+++
<i>H. anomala</i>	++	+++	—	+++	+	+++

indicates the effect of low concentrations of cobaltous acetate on certain yeasts that readily produced M structures on the potato agar control slide cultures. At 10<sup>-3</sup> M Co<sup>++</sup> there was a markedly more abundant production of M, with all the species tested, than was observed in the controls. In Table VII there is registered the effect of several heavy metals on some strains of yeasts that did not produce M under our control conditions.

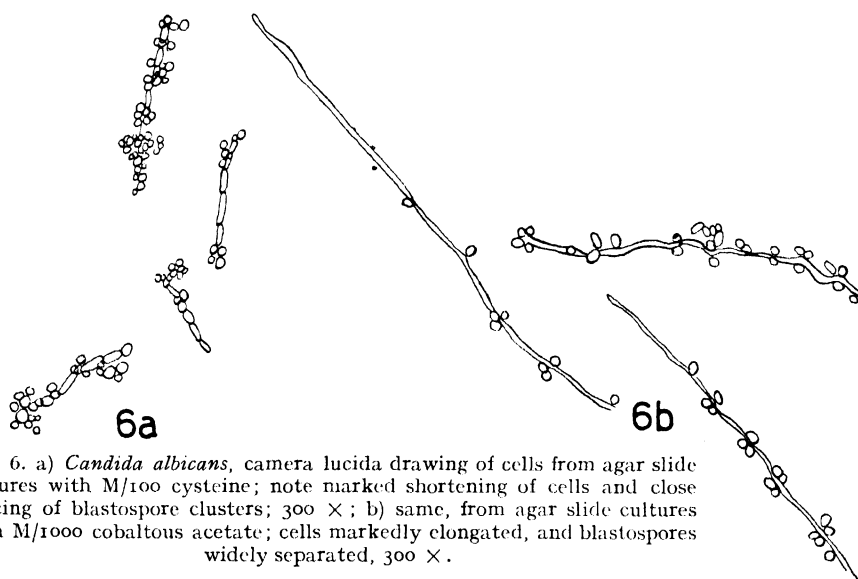


Fig. 6. a) *Candida albicans*, camera lucida drawing of cells from agar slide cultures with M/100 cysteine; note marked shortening of cells and close spacing of blastospore clusters; 300 ×; b) same, from agar slide cultures with M/1000 cobaltous acetate; cells markedly elongated, and blastospores widely separated, 300 ×.

A marked effect of 10<sup>-3</sup> M Co<sup>++</sup> is to be noticed. Aside from cobalt, only boron produced a significant effect on Y → M, though this effect was much less pronounced than that of cobalt. In Fig. 6 the shapes of cells of *C. albicans* from a 10<sup>-3</sup> M Co<sup>++</sup> slide culture are compared with those from a 10<sup>-2</sup> M cysteine slide culture; the cobalt-treated cells form mycelial elements — no cross walls were visible in the segments drawn. In contrast, the cysteine-treated cells, supporting blastospore structures, are extremely short.

#### Effect of Cobalt and Oxine on Yeasts on Solid and in Liquid Media

Recent publications by ALBERT *et al.*<sup>20</sup> have shown that cobalt is by far the most effective substance for reversing the growth inhibitory effect of oxine (8-hydroxy quinoline) on Gram + bacteria. We tried the effect of oxine on Y → M using the slide culture technique (Table VIII) but, at the concentrations employed, the most noticeable effect of oxine was on total growth. Experiments were then made on the effect of oxine

I  
ME YEASTS THAT DO NOT USUALLY PRODUCE M STRUCTURES

MnCl <sub>2</sub> (10 <sup>-3</sup> M)		Na <sub>2</sub> HAsO <sub>3</sub> (10 <sup>-3</sup> M)		Na <sub>3</sub> AsO <sub>4</sub> (10 <sup>-3</sup> M)		CoAc <sub>2</sub> (10 <sup>-3</sup> M)	
Y → M	Growth	Y → M	Growth	Y → M	Growth	Y → M	Growth
—	+++	—	+	—	—/+	+	+++
—	+++	—	+	—	++	+/-	+++
—	+++	—	+	—	—/+	—	+++
—	+++	—	+++	—	+	++	+++
++	+++	—	+	—	—/+	+++	+++

on the growth of *Candida albicans* (Gram+, as are all yeasts) in liquid media. A wort medium was prepared with additions as noted in Table IX; only at M/10000 oxine was an effect on growth detectable (ALBERT *et al.* reported M/80000 oxine prevented the growth of Gram+ bacteria; they, too, tested in a "natural" medium). Higher concentrations of oxine were therefore prepared (Table X) and inoculated; except for a moderate effect on growth when examined at 24 hours, oxine was not inhibitory at M/2500 concentration.

While it is clear that oxine is without appreciable effect itself, under our conditions, on Y → M or on total visible growth of yeasts in liquid media, it is also clear that it prevents the appearance of cobalt effects. Compare Y → M enhancement by cobalt, as shown in Tables VI and VII, with Table VIII. Cobalt has, moreover, been shown<sup>11</sup> to completely inhibit the growth of yeasts (*S. cerevisiae* and *C. albicans*) when added to natural media (beer wort) in concentrations greater than M/10000. Thus, oxine antagonizes both the growth-inhibitory and the Y → M promoting, effects of cobalt.

TABLE VIII  
EFFECT OF 8-HYDROXY QUINOLINE (OXINE) AND COBALT + OXINE ON Y → M

Organism	Control		Oxine (10 <sup>-4</sup> M)		Oxine (10 <sup>-4</sup> M) + CoAc <sub>2</sub> (2 · 10 <sup>-4</sup> M)	
	Y → M	Growth	Y → M	Growth	Y → M	Growth
<i>C. albicans</i> . . . .	+++	+++	+++	++	+/-	+++
<i>C. tropicalis</i> . . . .	+++	+++	++++	+/++	++++	++
<i>Trich. capitatum</i> . .	+++	+++	—	+/-	++	++
<i>H. anomala</i> . . . .	++	+++	+++	+++	++	++++

TABLE IX  
EFFECT OF 8-HYDROXY QUINOLINE (OXINE), COBALT, AND CYSTEINE ON THE GROWTH OF *Candida albicans* IN WORT; 22 HOURS INCUBATION AT 25° C

Addition Concentration	Oxine	Oxine (M = 1/x) + CoAc <sub>2</sub> (M = 2/x)	Oxine (M = 1/x) + cysteine (M/5000)	Oxine (M = 1/x) + CoAc <sub>2</sub> (M = 2/x) + cysteine (M/5000)
M/10000	+	++	+	+
M/25000	+++	+++	+++	+++
M/50000	+++	+++	+++	+++
M/80000	+++	+++	+++	+++

Blank (plain wort) = +++ ; Wort + M/5000 cysteine = +++

References p. 474/475.



TABLE X  
EFFECT OF 8-HYDROXY QUINOLINE ON THE GROWTH OF *Candida albicans* IN WORT AT 25° C

Concentration \ Addition	Oxine		Oxine (M = 1/x) + CoAc <sub>2</sub> (M = 2/x)		Oxine (M = 1/x) + CoAc <sub>2</sub> (M = 2/x) + cysteine (M/2 500)	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
M/2 500	+	+++	++	+++	++	+++
M/5 000	++	+++	+++	+++	++	+++
M/10 000	++	+++	+++	+++	+++	+++

Blank (plain wort) = +++ at 24 hours; Wort + M/2 500 cysteine = +++ at 24 hours

#### *Inhibition of Cell Division without Simultaneous Inhibition of Nuclear Division*

The processes involved in nuclear division may in many instances be regarded as precursors, in point of time, of the processes leading to the division of one cell into two. During Y → M conversions, however, it has been found that long M elements may exhibit several nuclei<sup>3</sup>, or only one or a few nuclei (Figs 7 and 8) along the length of an extensive filament. Evidently then, cell division may be inhibited under conditions that permit nuclear division to continue\*. LEVAN<sup>21</sup> described much the same situation



Fig. 7. *Candida albicans*, cells from control slide agar cultures stained with 1% aqueous toluidine blue (see text); 1000 ×, from 2 day culture.

\* Blastospores, which are produced from the M elements of *C. albicans*, *C. tropicalis*, *Trichosporum capitatum*, and many other yeasts, are seen (Figs 7–8) to be uninucleate Y entities. The morphogenetic phenomena underlying the intensive nuclear division which gives rise to a cluster of blastospores, localized at “nodes” spaced more or less regularly along an M filament, are worthy of serious study but are outside the scope of this paper. Blastospore production was rarely completely absent in these yeasts, even under the most pronounced Y → M stimulation, (Table VI, Fig. 6b); it should be noted, however, that at least the first blastospore produced at a given node must have derived its nucleus from the division of an M-nucleus.

from his study on the induction of aberrant forms in *S. cerevisiae* by treatment with camphor; nuclear division was found to proceed in the absence of cell division.

The appearance of the structure designated as a nucleus by us (Fig. 8) is fairly uniform from cell to cell. The cytological picture observed by us provides confirmation of the concept of the yeast nucleus presented by NAGEL<sup>22</sup>. Additional confirmation, by a completely different technique, has been supplied by the nuclear localization of yeast pyrophosphatase<sup>23</sup>.

The very low level of cytoplasmic basophilia in the yeasts grown on the potato agar medium seems worthy of note. Several of the species studied were grown in a wort medium and subsequently stained with toluidine blue as described; cells from such growths invariably showed such a dense overall staining that no internal detail could be discerned. As WIAME<sup>24</sup> and BRACHET<sup>25</sup> have pointed out, the affinity of a cell for toluidine blue is probably the result of its content of the components of a nucleic acid-metaphosphate complex. Metaphosphate is probably responsible for the metachromatic staining of yeasts as WIAME AND MICHAELIS<sup>26</sup>, have shown. By reason of the fairly firm cytochemical basis for the toluidine blue staining, we may infer that the

cells shown in Figs 7-8 have a very low cytoplasmic content of ribonucleic acid and metaphosphate. Such an interpretation is in line with the findings<sup>3</sup> that  $Y \rightarrow M$  is accompanied by a loss of Gram staining positivity and by a very greatly reduced affinity for pyronin from the Unna-Pappenheim stain. It is also important to note that PRATT AND DUFRENOY<sup>10</sup> have shown that  $B \rightarrow F$ , induced in Gram+ bacteria by penicillin, is accompanied by a loss of Gram positivity. Some implications of these cytochemical findings for the relationship between nucleic acids and the protein synthesis accompanying growth in the absence of cell division have already been indicated<sup>3</sup>. In contrast to these findings (based on the use of staining reactions) which indicate a lowered nucleic acid content in cells that have undergone elongation, HENRY *et al.*<sup>27</sup> found, by analytical determination, that no change was produced in the total amount of nucleic acid, or in the ratio of the two types of nucleic acid, in cells of *Clostridium Welchii* in which elongation had been induced by sub-bacteriostatic concentrations of penicillin. On the other hand, they obtained<sup>27</sup> certain of the protein constituents of the cytoskeleton in unusual fibrous form. Their findings are not necessarily in contradiction to the results achieved with staining procedures, which probably depend upon the physical state of nucleate-protein linkages (through -SH protein bonds) in the case of the Gram stain<sup>28</sup>, and probably upon the degree of polymerization of ribonucleic acid in the case of the basophilic stains. The parallel between prevention of fibrous protein formation and the

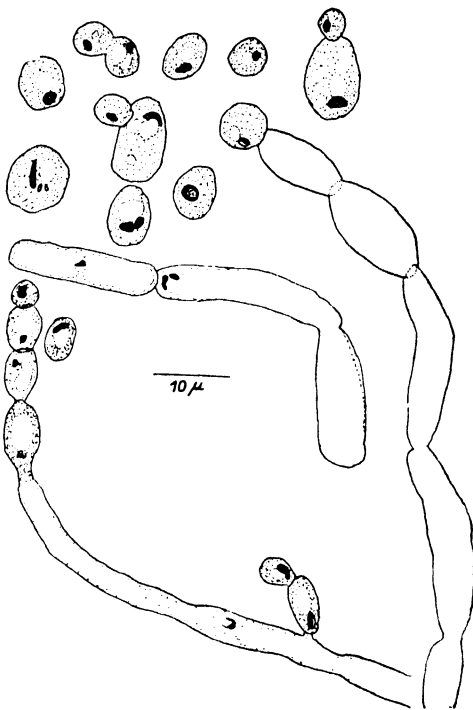


Fig. 8. As Fig. 7, from 5 day old culture

maintenance of cell division has already been discussed<sup>3</sup>, see also<sup>7</sup>. In this connection it is of interest to note that LWOFF AND JONESCO<sup>37</sup>, with *Moraxella Lwoffii*, have obtained extensive elongation of the nuclear apparatus accompanying filament formation, induced by cultivation of the bacterium in the absence of potassium.

#### DISCUSSION

The importance of sulfhydryl groups in the cell division processes of microorganisms, as demonstrated in this paper, is, we believe, the first demonstration of the importance of such groups in the cell *division* processes, as distinguished from the total growth processes involved in cell *multiplication*. The importance of -SH groups for cell multiplication processes in yeasts has been known since RAPKINE<sup>8</sup> inhibited multiplication in *Schizosaccharomyces pombe* with dilute iodoacetate and overcame the inhibition with glutathione; fermentation was also inhibited by iodoacetate at the concentrations inhibiting multiplication. In our experiments energy yielding processes, sufficient to support growth, are not inhibited when the cell division mechanism is affected. While a great many experiments, on a variety of biological forms, have demonstrated the undeniable importance of sulfhydryl group-substances in cell multiplication, it must be emphasized that most experiments have not distinguished between effects on cell division *per se* and effects on growth (defined as an irreversible increase in volume). With the relatively favourable material at our disposal we may say that these initial experiments indicate very strongly that *cell division, as a process distinct from growth, depends upon the maintenance of functional intracellular sulfhydryl groups.*

The relationship of the phenomenon we designate as  $Y \rightarrow M$  to the "camphor-reaction" of yeasts as reported by BAUCH<sup>29, 30</sup>, LEVAN<sup>21, 31</sup>, and SKOVSTED<sup>33</sup> requires consideration. Camphor, and numerous other water-insoluble substances, have been shown to induce the development of rather characteristic morphological irregularities during the growth of *Saccharomyces cerevisiae*. Elongated cells with undulating contours, and a marked tendency to remain attached in chains, have been induced in yeasts by the action of camphor. LEVAN<sup>21</sup> has made an extensive study of chemical structure *vs* "camphor-reaction" activity with *S. cerevisiae*; over 50 compounds, chiefly known narcotizing agents, were found to be effective. A pronounced parallel, within an homologous series, could be noted between activity and increasing insolubility in water; activity was believed to reside in relatively non-specific surface-acting properties of the compounds. Such action is in contrast to the relatively specific action of the compounds we have studied which are effective in high dilution and involve sulfhydryl groups. It seems entirely possible, however, judging from the similarities in the results effected by the two classes of agents, that we are witnessing two modes of attack on the cell division mechanism: (1) the water soluble, -SH specific class (exemplified by penicillin) directly affecting the cell division enzyme mechanism, and (2) the water insoluble, non-specific, surface active narcotics (exemplified by camphor) affecting the formation of new, discontinuous cellular phase boundaries by interference at the "product end" of the division mechanism. The discussion by VELDSTRA<sup>34</sup> of the mode of action of non-polar ergons is pertinent to our consideration of the existence of two modes of chemical attack on the cell division mechanism. From the facts amassed by VELDSTRA it seems clear that we may expect a relative non-specificity among the lipophilic substances studied by LEVAN, and for which we may also expect an effect on protoplasmic boundary

systems (apparently interference with protoplasmic boundary formation in the present instance).

In his work on camphor-treated yeasts BAUCH observed a primary reaction, essentially as outlined above, and a secondary permanent cell modification which he regarded as a colchicine-type, chemically-induced polyploidy because of the permanently increased cell volume. SKOVSTED has reviewed the literature on the induction of polyploidy in yeasts by treatment in this manner, and concluded the question is not settled. It is clear from SKOVSTED's work, however, that camphor treatment leads to an increased rate of mutation in yeasts, as well as to temporary morphological modification. It is becoming clear that substances inhibiting cell division in micro-organisms and the mitotic poisons of higher plants and animals (colchicine-type and "radiomimetic") have some interesting common aspects. As DUSTIN<sup>35</sup> points out, "while mitosis may be affected in several ways, the one important thing is that we have many substances that specifically affect dividing cells". He has suggested enzyme inhibition as a mode of action of mitotic poisons, including radiation effects as well as chemical mitotic poisons<sup>36</sup>. The sulfhydryl-enzyme-inhibiting properties of X-rays and of many of the mitotic poisons are well known. In comparison, our data permit the conclusion that maintenance of intracellular -SH groups is a necessity for cell division processes, and that their temporary inhibition by -SH enzyme-specific substances leads to temporary morphological modification as a result of cell division inhibition.

#### Acknowledgment

The authors wish to express their gratitude to Professor A. J. KLUYVER for his encouragement and interest in this work.

#### SUMMARY

A concept is developed regarding the occurrence of yeast to mycelia conversions ( $Y \rightarrow M$ ), resulting in the appearance of filaments from single cell yeasts, as the differential inhibition of cell division processes without the simultaneous inhibition of growth processes (defining growth as an irreversible increase in volume).

Sulfhydryl group substances, such as cysteine and glutathione, are shown to promote cell division and to inhibit the appearance of  $Y \rightarrow M$  in organisms that regularly produce M structures. Ascorbic acid is without such effect. It is suggested that the metabolically controlled intracellular sulfhydryl  $\rightleftharpoons$  disulfide equilibria may reasonably account for many of the observations in the literature concerning the influence of the growth medium on the dimorphism ( $Y \rightarrow M$ ) of *Candida albicans* (and other species of the *Mycotoruloideae*).

Inhibition of cell division (and stimulation of  $Y \rightarrow M$ ) is shown to result from the exposure of different species of yeasts to penicillin and to cobaltous ions. With the exception of boron (slight effect), other heavy metals examined were without such a differential effect. The effect of penicillin and cobalt was antagonized by cysteine.

Preliminary cytological studies (employing toluidine blue and cells with a greatly reduced cytoplasmic basophily) indicate that nuclear division may proceed even though cell division stops. This results in the occurrence of multinucleate M elements, as a result of  $Y \rightarrow M$ .

The findings are considered in relation to some general aspects of cell division, including comparisons with the effect of camphor on yeasts and the action of mitotic poisons.

#### RÉSUMÉ

Une hypothèse est discutée concernant la transformation de la forme levure en forme mycélium ( $Y \rightarrow M$ ), cette transformation étant due à l'inhibition spécifique des processus de division cellulaire sans l'inhibition simultanée des processus de croissance (la croissance étant définie comme une augmentation irréversible de volume).

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Les substances à fonction SH telles que la cystéine et le glutathion provoquent la division cellulaire et inhibent le passage  $Y \rightarrow M$  chez les organismes qui produisent régulièrement la forme M. L'acide ascorbique n'a aucune action. On imagine que l'équilibre intracellulaire sulphydryle  $\rightleftharpoons$  disulfure peut raisonnablement rendre compte de la plupart des observations concernant l'influence du milieu de croissance sur le dimorphisme ( $Y \rightarrow M$ ) de *Candida albicans* (et d'autres espèces de *Mycotululoideae*).

L'inhibition de la division cellulaire (et la stimulation de  $Y \rightarrow M$ ) est provoquée par le traitement de différentes espèces de levures par la pénicilline et par les ions cobaltux. Sauf en ce qui concerne le bore (effet léger) les autres métaux lourds étudiés ne présentent pas d'action. L'effet de la pénicilline et du cobalt est empêché par la cystéine.

Des études cytologiques préliminaires (à l'aide de bleu de toluidine) montrent que la division du noyau peut avoir lieu même lorsque la division cellulaire est bloquée. Ceci provoque l'apparition d'éléments M multinucléés comme on les trouve dans  $Y \rightarrow M$ .

Ces observations sont discutées en relation avec quelques aspects généraux de la division cellulaire, en particulier l'action du camphre sur les levures et l'action des poisons de la mitose.

### ZUSAMMENFASSUNG

Eine Hypothese über die Verwandlung der Hefezellen in ein Hefemycelium ( $Y \rightarrow M$ ) wird aufgestellt. Diese Verwandlung ist auf eine spezifische Hemmung der Zellteilung ohne gleichzeitige Hemmung des Wachstums zurückzuführen, wenn man das Wachstum als eine irreversible Zunahme des Volumens definiert.

Verbindungen die wie Cystein oder Glutathion SH-Gruppen enthalten, rufen Zellteilungen hervor und hemmen die Verwandlung  $Y \rightarrow M$  bei Organismen die regelmässig Mycelien bilden. Ascorbinsäure hat keine derartige Wirkung. Die Verfasser nehmen an, dass das in der Zelle herrschende Gleichgewicht Sulphydryl  $\rightleftharpoons$  Disulfid die meisten Beobachtungen über den Einfluss des Nährbodens auf den Dimorphismus ( $Y \rightarrow M$ ) von *Candida albicans* (und anderer Mycotululoideae-Arten) erklären kann.

Es wird gezeigt dass Hemmung der Zellteilung (und von  $Y \rightarrow M$ ) durch Behandlung verschiedener Hefearten mit Penicillin und mit  $Co^{++}$  bewirkt wird. Mit Ausnahme von Bor (das eine schwache Wirkung hat) zeigen die anderen untersuchten Schwermetalle keine Wirkung. Die Wirkung von Penicillin und  $Co^{++}$  wird durch Cystein gehemmt.

Cytologische Vorversuche (mit Toluidinblau) lehren, dass die Kernteilung vor sich gehen kann, auch wenn die Zellteilung aufhört. Das Ergebnis sind Mycelien mit mehreren Kernen, wie man sie in  $Y \rightarrow M$  antrifft.

Diese Ergebnisse werden im Hinblick auf einige allgemeine Aspekte der Zellteilung insbesondere den Effekt von Kampfer auf Hefe und die Wirkung der Mitosegifte erörtert.

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## ACCUMULATION OF RADIOACTIVE COBALT BY DIVIDING YEAST CELLS

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### INTRODUCTION

Until recently the literature on the metabolism of cobalt by microorganisms was limited to a few studies wherein it had been included in investigations of the toxicity of a series of cations<sup>1, 2</sup>. While the wide-spread distribution of cobalt in soils, and its general distribution in the tissues of higher plants<sup>3, 4</sup>, and in animal tissues<sup>5</sup> are well known, there seems to be no report of the natural occurrence of cobalt in the cells of microorganisms. Spectroscopic examination of the ash of yeast cells failed to reveal the presence of cobalt<sup>6</sup>. In contrast to the findings with some other metals (*i.e.*, Zn, Cu, Fe, Mn, Mo, Ga, and Tl), trace concentrations of which are known to stimulate the growth of different microorganisms, there seems to be no report for any such action on the part of ionic cobalt. Beginning with very dilute concentrations, cobalt is reported to have the effect of progressively decreasing the amount of growth of different microorganisms when added to culture media in increasing concentration<sup>1, 7</sup>. With some animals, on the contrary, cobalt is known to be an essential trace element (for review, see<sup>8</sup>); a deficiency of cobalt in pasturage has been shown to be responsible for the appearance of characteristic symptoms in ruminants feeding thereon, the condition having been recognized in several localized areas throughout the world.

Within the past year work has appeared from different laboratories indicating that cobalt is probably of especial importance in the metabolism of several groups of microorganisms. ALBERT *et al.*<sup>9</sup> have shown that cobalt is specifically active in reversing the inhibitory action of oxine (8-hydroxy quinoline) on Gram positive bacteria, (while zinc and iron have such an action with Gram negative bacteria). In contrast, PRATT AND DUFRENOY<sup>10</sup> have observed that cobalt specifically potentiates the *in vitro* and *in vivo* action of penicillin. NICKERSON AND VAN RIJ<sup>11, 12</sup> have found that cobalt can selectively inhibit cell division in various yeasts without simultaneously inhibiting growth (defined as an irreversible increase in volume) and other metabolic processes. Thus, they have obtained the growth of yeasts as elongated mycelial elements. The selective action of

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cobalt against the enzymatic mechanism of cell division<sup>11</sup> was observed to be specifically antagonized by sulphhydryl group compounds.

In the present study we have examined quantitatively the relationship between cobalt concentration and the growth of yeasts (measured as weight increase) in liquid media. Employing radioactive cobalt, the extent to which yeast cells accumulate this ion has been determined. A preliminary fractionation has been made to learn the manner in which cobalt is bound in the yeast cell. A possible mechanism by which yeast cells may concentrate cobalt from dilute solutions is briefly considered.

#### MATERIALS AND METHODS

Pure cultures of two different yeasts were employed; *Saccharomyces cerevisiae* (Carlsberg No. 237), and a pathogenic non sporulating yeast, *Candida albicans*. Stock cultures were maintained in 8% Pilsner wort in Freudenreich flasks at 25°. For experimental purposes the yeasts were grown in a medium (GGY) consisting of: 20.0 g glucose, 10.0 g glycine, 1.0 g yeast extract (Difco), and one liter glass distilled water. To this medium appropriate dilutions of a solution of cobaltous nitrate (Merck, Darmstadt,  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ) were added to give final concentrations of  $\text{Co}^{++}$  ranging from M/1000 to M/64000. The medium was dispensed (50 ml per 100 ml Erlenmeyer flask), autoclaved (120° for 20 min), cooled, and inoculated with 0.1 ml of a standard density suspension of washed cells obtained from a 24 hour growth. The flasks were then incubated, with or without continuous agitation, as indicated. Growth measurements were made by removing 10 ml aliquots, in duplicate, from duplicate flasks, filtering through tared Schott G-4 fritted glass filters, washing with distilled water, drying at 105° for 16 hours, and weighing. Duplicate determinations by this method agreed to within  $\pm 0.2$  mg.

Radioactive cobalt (as cobaltous nitrate) was incorporated into 500 ml quantities of the GGY medium contained in one liter Florence flasks, inoculated, and incubated with continuous agitation for 17 hours at 20°. Samples of the yeast crop (obtained from the centrifuged, triply-washed, yeast cream), as well as the samples obtained by subsequent fractionation procedures, were placed on aluminum dishes adapted for GEIGER-MÜLLER counting, and measured automatically<sup>13</sup>. The radioactive cobalt was obtained as a purified solution of cobalt nitrate; it comprised a mixture of isotopes of cobalt with an average half-life of about 78 days.

#### EXPERIMENTAL

##### *Cobalt Concentration vs Yeast Growth*

The inhibitory effect of cobalt on the growth of yeast in a natural medium was apparently first studied by BOKORNY<sup>1</sup>. He observed no visible growth of yeast with concentrations of  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  greater than 0.02% ( $7 \cdot 10^{-4}$  M). This concentration limit agrees well with our own findings. Results on the growth of *S. cerevisiae* in two series of experiments with different cobalt concentrations are given in Table I. Culture medium with a cobalt ion concentration greater than  $10^{-4}$  M permitted little, or no, growth of the yeast. A rather sharp break in the curve of growth vs cobalt concentration in the region of  $10^{-4}$  M  $\text{Co}^{++}$  was apparent. No concentration of cobalt tested was found to increase the growth of *S. cerevisiae*, thus agreeing with the report of MOKRAGNATZ<sup>7</sup>, made on *Aspergillus niger*.

BURK *et al.*<sup>14, 15</sup> observed an inhibitory effect of cobalt on the growth and respiration of various microorganisms; this inhibition could be reversibly overcome by the addition of histidine, which was shown<sup>16</sup> to form a chelate complex with cobalt. The cobalt inhibition of yeast growth has been found to be antagonized by oxine<sup>12</sup>; oxine itself was without appreciable effect on the strains of yeasts employed. Another type of cobalt complex (ammine complexes) was shown by BOOI<sup>2</sup> to be without inhibitory effect on yeast fermentation, even at a concentration of 0.1 M, whereas  $\text{Co}^{++}$  caused a very marked



TABLE I

EFFECT OF COBALT CONCENTRATION ON THE GROWTH OF *Saccharomyces cerevisiae*  
DATA PRESENTED AS mg DRY WEIGHT OF YEAST PER 50 ml CULTURE; AVERAGE OF DUPLICATES

Series	Culture Conditions	Cobalt Concentration								
		M/1 000	M/2 000	M/4 000	M/8 000	M/16 000	M/32 000	M/40 000	M/64 000	Zero
A	Constant agitation at 20° for 48 hours	< 0.1	0.1	0.3	—	—	—	26.8	—	64.8
B	Constant agitation at 20° for 24 hours	—	—	1.4	4.2	10.7	17.8	—	39.8	53.0
C	Stationary culture at 25° for 24 hours	—	—	1.3	3.2	16.0	40.6	—	42.7	50.3

inhibition of fermentation at 0.01 M. The experiments of BOOIJ were conducted with non-dividing cells, and the concentration of  $\text{Co}^{++}$  necessary to achieve inhibitory effects was about  $10^2$  greater than we have found necessary to cause almost complete inhibition of growth in liquid media. Likewise, the effect of cobalt on yeast cells growing in liquid media is somewhat different from its effect on cells growing on the surface of agar media. Concentrations of  $10^{-3}$  M and  $5 \cdot 10^{-2}$  M  $\text{Co}^{++}$  cause no appreciable inhibition of the amount of yeast growth on agar media, but do result in the inhibition of cell division and the consequent growth of the yeast in a filamentous manner. The effect of cobalt on the morphology of yeast grown in liquid media is not so pronounced. The differing sensitivity of yeasts to cobalt when grown in the two types of media may result in part from differences in oxygen tension; reaction of the cobalt with some component of the agar also appears to be a possibility.

#### *Accumulation of Radioactive Cobalt by Growing Yeasts*

Radioactive cobalt incorporated into the GGY medium was found to be taken up by yeast cells during growth and to become more concentrated in the cells than in their growth medium. The accumulation of cobalt by the cells represented a many-fold concentration of the ion intracellularly compared with the concentration initially present in the medium. Data in this respect, for *S. cerevisiae* and for *C. albicans*, are shown in Table II. The strain of *S. cerevisiae* employed accumulated a cobalt concentration approximately 670-fold that present in the medium, while the cells of *C. albicans* achieved a 25-fold concentration (the *C. albicans* culture was not aerated, however).

The cobalt taken up by the cells was not loosely adsorbed to the cell surface since there was no detectable loss of activity by the cells after shaking them for 20 hours in sterile distilled water at 20° (Table III). If physical adsorption at the cell surface is involved in cobalt retention by these yeasts, the binding forces must not be overcome by the washing procedure employed. It is also evident that the cobalt accumulated by the cells is not free to diffuse out of the cells.

Water-washed yeast was subjected to continuous extraction with ether-acetone (1:1) in a Soxhlet apparatus for 24 hours. Activity measurements were made on the acetone-ether extract (directly, and after concentration), and on the residual yeast mass. The yeast before extraction contained 79.3% water; the extraction removed

TABLE II  
ACCUMULATION OF LABELLED COBALT BY YEASTS DURING GROWTH

Culture Conditions	Organism and Fraction	Counts per minute	Counts/min/g wet wt	Activity in cells Activity in medium
Constant agitation at 20° C for 17 h in 600 ml of medium; 21.0 µg/L initial cobalt conc., 106 mg dry wt yeast measured for activity	<i>S. cerevisiae</i>			
	yeast cells	4360.	8700.	—
	medium-fresh	65/ml	65.	134
	medium-spent	44.4/ml	44.4	196
Constant agitation at 20° C for 66 h in 1000 ml of medium; 62.5 µg/L initial cobalt conc., 60 mg dry wt yeast measured for activity	<i>S. cerevisiae</i>			
	yeast cells	6243.	21390.	—
	medium-fresh	190.6/ml	190.6	112
	medium-spent	31.9/ml	31.9	670
Stationary culture at 25° C for 168 h in 400 ml of medium; 21.0 µg/L initial cobalt conc., 278 mg dry wt yeast measured for activity	<i>C. albicans</i>			
	yeast cells	2083.	1500.	—
	medium-fresh	70/ml	70.	21
	medium-spent	60/ml	60.	25

TABLE III

RETENTION OF LABELLED COBALT BY CELLS OF *C. albicans* SUBJECTED TO WASHING WITH DISTILLED WATER FOR 20 HOURS AT 20°

Cells employed were part of the crop grown for Table II.

Fraction	Dry wt of samples (mg)	Counts/min	Counts/min/g wet wt
Yeast cells (after washing)	203	1660	1600
Wash water (60 ml)	—	< 1.5	< 1.5

TABLE IV

EXTRACTION OF LABELLED COBALT FROM CELLS OF *S. cerevisiae*

One liter culture incubated 66 hours at 20° with continuous agitation; 20.7 micro-Curies (62.5 micrograms cobalt) added per liter. See Table II for details on total crop.

Fraction	% Standard	% Standard/gm yeast wet wt
Whole yeast . . . . .	308/60 mg dry wt	1030
Ether extract . . . . .	0.05/ml	zero
Yeast (ether extracted) . . . . .	531/143 mg dry wt	745
Trichloroacetic acid extract (filtrate after neutralization) . . . . .	26/ml	200
Trichloroacetic acid extract (precipitate after neutralization)	250/19 mg dry wt	700
Yeast (trichloroacetic acid extracted) . . . . .	13.2/78 mg dry wt	35
Wash water used on acid extracted yeast . . . . .	2.53/ml	100

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81.4% of the fresh weight, and 10.0% of the dry weight of the yeast. Results of the activity measurements are shown in Table IV; it is clear the cobalt was not removed from the yeasts by the acetone-ether extraction.

The residual yeast mass from the above extractions was subsequently extracted with cold, 10% trichloroacetic acid; insoluble material was removed by centrifugation and washed with water. A portion of the acid extract was neutralized with NaOH, and allowed to stand. A flocculant, brownish precipitate settled from the neutralized extract; it was removed by filtration, and both filtrate and precipitate were measured for activity. The residue insoluble in trichloroacetic acid, and the water used in washing it by suspension and centrifugation were also measured for activity; results shown in Table IV. It is clear that the trichloroacetic acid extraction removed the greater part of the cobalt activity from the yeast, and that most of the activity in the extract was precipitated on neutralization.

Since inorganic cobalt ( $\text{Co}^{++}$ ), if present in the acid extract, would be precipitated on neutralization by NaOH, we carried out a neutralization in another experiment with  $\text{NH}_4\text{OH}$ , which leads to the formation of soluble ammine-complexes and provides a partial means for distinguishing between the possibilities of an organic or an inorganic binding of the cobalt in the acid extract. In this experiment inactive carrier cobalt was added to a trichloroacetic acid extract which was then divided into two equal volumes. One volume was neutralized with NaOH, and the other with  $\text{NH}_4\text{OH}$ . A slight, brownish, flocculent precipitate still developed in the extract neutralized with  $\text{NH}_4\text{OH}$ ; the precipitate with NaOH was in this case heavier and bluish-green, due to the presence of the added carrier cobalt. Activity measurements on the two precipitates (total amount in each case) gave nearly identical values: 55% standard with  $\text{NH}_4\text{OH}$ , and 43% standard with NaOH neutralization. Unfortunately, because of lack of sufficient radioactive cobalt, we have been unable to repeat this experiment. We can only conclude that it *indicates* that the cobalt activity in the trichloroacetic acid extract was not in ionic form, but probably in some organic combination in which the cobalt is so held that it did not exchange with added inorganic cobalt ions.

#### DISCUSSION

The avidity of growing yeast cells for the cobaltous ion, as shown in our experiments, is in marked contrast to the fate of cobalt injected into animals; yet cobalt is toxic for yeasts, and an essential trace element rôle in animals, at least in ruminants. COPP AND GREENBERG<sup>17</sup> found that 90% of the radioactive cobalt injected intraperitoneally into rats was excreted within four days. HEVESY<sup>18</sup> found no uptake of radioactive cobalt by red blood corpuscles. Oral administration of labelled cobalt to rats resulted in 80% elimination in the feces, 10% rapidly eliminated in the urine, and very little retention by tissues; only the liver consistently accumulated significant amounts<sup>19</sup>. Injection of cobalt has been found not to cure "coast" disease in sheep; cure is accomplished only on feeding cobalt<sup>20</sup>; the suggestion has been made that ingested cobalt may act upon microorganisms of the rumen and, not directly, upon the host. Certainly, our own results show that furnishing cobalt to two different microorganisms results in their accumulating and combining it.

The occurrence of cobalt in an organic compound of biological origin was reported<sup>21, 22</sup> simultaneously with our first notice<sup>11</sup> of the possible organic combination of the labelled

cobalt extracted from yeast cells. The demonstration<sup>21, 22</sup> of the presence of cobalt in the anti-pernicious anemia factor raises still more interest in the problem of cobalt metabolism by animals, plants, and microorganisms. The existence of a requirement for an organic cobalt compound (vitamin B<sub>12</sub>, the anti-pernicious anemia factor) by a microorganism, *Lactobacillus lactis*<sup>23, 24</sup>, which cannot be satisfied by supplying the cobalt ion<sup>22</sup>, is now known. Demonstration of whether this is a true requirement for organically bound cobalt, or merely for the organic part of the molecule is yet to be made. The complete absence of requirement for a cobalt compound or ionic cobalt in the case of a yeast, *S. cerevisiae*, is indicated by the report<sup>6</sup> of the absence of cobalt in yeast ash examined spectroscopically; the absence of cobalt in the ash of the plant extract medium (malt extract) used to support the growth of the yeasts in these experiments<sup>6</sup> should also be noted.

The mechanisms by which microorganisms (including plankton in the sea) are able to accumulate significant concentrations of various cations from extremely dilute solutions are of considerable interest\*. An attractive suggestion has recently been made by HUTNER<sup>25</sup> that the cell surface of a microorganism may include among its architectural features the presence of a variety of compounds capable of acting as metal receptors by reactions of a chelating nature. The effectiveness of many substances, acting by chelation, for quantitative reaction with cations is well known. We have considered the possibility that accumulation of cobalt by yeast cells may be the result of its combination with substances at the cell surface. One substance in yeast cells that is known to form complexes with metals is metaphosphate. A peripheral distribution for this substance in yeast cells is a likely possibility, judging from staining reactions<sup>26</sup>, and from the localization of alkaline hexametaphosphatase in yeasts<sup>27</sup>. Cobalt is not precipitated under alkaline conditions when hexametaphosphate is present, indicating suppression of Co<sup>++</sup> by metaphosphate\*\*. Complex formation by metaphosphate with Ca<sup>++</sup>, Mg<sup>++</sup>, and Fe<sup>+++</sup> has been discussed by QUIMBY<sup>28</sup>. It is suggested that peripherally located metaphosphate might be of significance in the accumulation of metals by yeasts from dilute solutions.

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#### SUMMARY

Data are presented on the relationship between cobalt concentration in a nutrient medium and the growth of yeast cells. No evidence was found for growth stimulation at dilute cobalt concentrations. The toxicity of cobalt for yeast growth increases with increasing concentration, and becomes markedly toxic in the region of 10<sup>-4</sup> M Co.

\* With extremely dilute labelled copper (10<sup>-10</sup> M), MAZIA AND MULLINS<sup>30</sup> observed an almost quantitative removal of the Cu<sup>++</sup> by photosynthesizing leaves of *Elodea* (a concentration of 3 · 10<sup>8</sup> of Cu<sup>++</sup> inside over outside was obtained).

\*\* Systems of FeCl<sub>3</sub>-Co(NO<sub>3</sub>)<sub>2</sub>-Na hexametaphosphate and FeCl<sub>3</sub>-Co(NO<sub>3</sub>)<sub>2</sub>-oxine have been examined<sup>29</sup>; it has been found that cobalt weakens the iron-metaphosphate and iron-chelate complexes with the reappearance of Fe<sup>+++</sup>. The possibility of an action of this type being a basis for iron "mobilization" (e.g., action of one trace metal in promoting the formation of an organic complex of another trace metal, *ex.*, action of Cobalt on hemoglobin formation) has been considered.

Uptake of labelled cobalt by growing yeasts in the accumulation of cobalt by the cells to a level over 600 times greater than the concentration initially present in the medium. The cobalt accumulated by the cells was not free to diffuse out of the cells when subjected to prolonged washing in distilled water. Acetone-ether extraction of water washed cells removed over 80% of the fresh weight, and 10% of the dry weight of the cells but removed no labelled cobalt. The cobalt was largely extracted from the cells by cold 10% trichloroacetic acid, from which it precipitated as organically combined cobalt on neutralization of the acid extract.

The ability of yeast cells to accumulate cobalt from dilute solutions during growth is suggested to result from the presence of ion-suppressing, complex-forming substances (such as metaphosphate) at the cell surface which can unite with cobalt to effect a nearly quantitative removal of the ion from a dilute solution.

Some aspects of cobalt metabolism are briefly discussed.

## RÉSUMÉ

Les résultats obtenus concernent les relations existant entre la concentration en cobalt dans un milieu de culture et la croissance des cellules de levure. Il n'apparaît aucune stimulation de la croissance aux concentrations diluées en cobalt. La toxicité du cobalt pour la croissance des levures s'accroît avec la concentration et devient très nette pour une concentration de l'ordre de  $10^{-4}$  M.

Les levures en voie de croissance accumulent du cobalt marqué jusqu'à une concentration plus de 600 fois supérieure à la concentration initiale du milieu. Le cobalt ainsi concentré dans les cellules ne peut plus diffuser à l'extérieur lorsqu'on lave les cellules d'une façon prolongée par l'eau distillée. Le traitement des cellules par l'acétone-éther élimine plus de 80% du poids frais des cellules déjà lavées par l'eau, et 10% du poids sec, mais n'enlève pas de cobalt marqué. Au contraire le cobalt est extrait pour la plus grande part par l'acide trichloroacétique à 10% dont on peut le précipiter sous forme organique par neutralisation de l'extrait acide.

L'aptitude des cellules de levures à accumuler le cobalt à partir de solutions diluées au cours de la croissance semble due à la présence de substances capables de former des complexes (telles que le métaphosphate) à la surface de la cellule, de telle sorte que la fixation du cobalt soit pratiquement quantitative. Quelques aspects du métabolisme du cobalt sont brièvement discutés.

## ZUSAMMENFASSUNG

Die beschriebenen Versuchsergebnisse betreffen das Verhältnis zwischen der Kobaltkonzentration im Nährboden und dem Wachstum der Hefezellen. Bei geringen Kobaltkonzentrationen konnte keine Wachstumsförderung festgestellt werden. Die Giftigkeit von Kobalt für Hefezellen nimmt mit der Konzentration zu und wird bei einer Konzentration von  $10^{-4}$  M Co sehr deutlich.

Wachsende Hefe sammelt markiertes Kobalt bis zu einer Konzentration die 600 mal grösser ist, als die Anfangskonzentration im Nährboden, in den Zellen an. Das so angesammelte Kobalt kann auch bei langem Waschen mit destilliertem Wasser nicht mehr aus den Zellen herausdiffundieren. Werden die Zellen nach dem Waschen mit Wasser mit Aceton-Äther extrahiert, so werden über 80% des Frischgewichtes und 10% des Trockengewichtes aber kein markiertes Kobalt entfernt. Dagegen wurde das Kobalt durch kalte 10%-ige Trichloressigsäure weitgehend extrahiert. Aus dieser Lösung kann es als organisches Kobalt durch Neutralisation des sauren Extraktes gefällt werden.

Die Fähigkeit der Hefezellen Kobalt aus verdünnten Lösungen während des Wachstums anzusammeln, scheint von komplexbildenden Substanzen (wie Metaphosphat) herzurühren die sich an der Oberfläche der Zelle befinden und sich mit dem Kobalt verbinden so dass dieses Ion nahezu quantitativ aus verdünnten Lösungen entfernt wird.

Einige Aspekte des Kobaltmetabolismus werden kurz auseinandergesetzt.

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## THE CHANGES IN THE ELECTRIC IMPEDANCE DURING ACTIVITY AND THE EFFECT OF ALKALOIDS AND POLARIZATION UPON THE BIOELECTRIC PROCESSES IN THE MYELINATED NERVE FIBRE

by

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In 1939, COLE AND CURTIS made alternating current impedance measurements on the fresh water plant *Nitella* and the giant axon of the squid during the passage of an impulse. In their experiments, the relationship between the membrane resistance and the electromotive force therein has been most impressively brought out. Hence a similar experiment on the myelinated nerve fibre seemed very desirable to us for a better understanding of the process of excitation in the plasma membrane at the node of RANVIER.

In the present work, the impedance of the single motor nerve fibre of the toad has been investigated by the use of the "bridge-insulator" method. This method of insulating an isolated single nerve fibre between two neighbouring nodes provides us with a simple but very direct way of demonstrating the remarkably close interrelationship between the impedance changes and the action current in this fibre. The effects of several alkaloids and electrotonic polarization upon the time course of the impedance changes have further been investigated in the hope of securing additional evidence that the impedance change and the production of action current are only two different expressions of one and the same process occurring at the plasma membrane of the node of RANVIER.

With a whole nerve trunk, LULLIES (1930) has already found a decrease in the impedance for bridge currents strong enough to excite the nerve fibres. But, as the effect is demonstrable with only low frequency alternating currents, its relationship with those presented in this paper is not very clear at present.

### METHOD

Nerve fibres of above 11 microns in diameter have been selected for the experiments. The technique of isolating the fibres is fundamentally the same as that described previously (TASAKI, 1939), except that in the present investigation the operation was carried out under a binocular microscope. Care was taken to remove all the inactive fibres and tissues around the nerve fibre to be examined. The isolated region of the nerve fibre was mounted on a "bridge-insulator" consisting of two separate glass plates fixed at a distance (between the edges) of about 1 mm. In each of the pools of RINGER on both sides of the bridge-insulator, a non-polarizable electrode of the  $\text{Zn-ZnSO}_4$ -RINGER (agar) type was immersed.

The isolated region of the fibre on the bridge-insulator, together with the electrodes dipping into the pools, formed one arm of a Wheatstone bridge. The experimental arrangement used in the later stage of the present investigation is shown in Fig. 1. The resistance  $R$  in the figure was in most cases 1 megohm and  $r$  was generally between 0.3 and 3 ohms. The bridge was balanced by adjusting the condenser  $C$  and the resistance  $r$  continuously. The bridge input consisted of an alternating

current (A.C.) from a beat-oscillator, its frequency being between 1 and 10 kilocycles per second (kc). As too strong an A.C. reduced the electric response of the nerve fibre, as already described by ROSENBLUTH (1940) and his coworkers care was taken not to allow its amplitude to exceed about 50 mV.

Most experiments were made at temperatures between 2 and 6° C. At such low temperatures the duration of the action current of the nerve fibre is several msec and this made it possible to demonstrate impedance changes with A.C. of relatively low frequencies. Moreover, cold raises the threshold of the fibre for brief currents and consequently permits us to increase the bridge input without appreciably affecting the excitability of the fibre. At 2° C, an A.C. of about 20 mV at 3 kc was found not to bring about any detectable change in the threshold of the fibre.

The nerve fibre was brought into action by means of an induction coil I or a battery B in the figure. The contacts in the primary circuit of the induction coil and in the battery circuit ( $K_1$  and  $K_2$ ) were operated with a HELMHOLTZ pendulum, of which another break contact was used to start single sweeps of the cathode ray oscillograph. The figures on the face of the oscillograph were photographed.

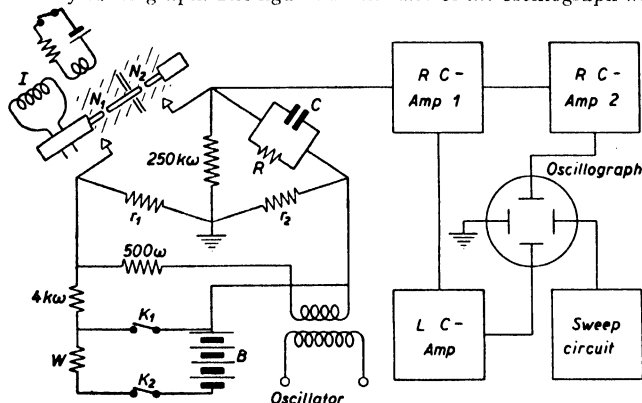


Fig. 1. Schematic diagram of the electrical equipment used for the impedance determinations during activity. The nerve fibre, of which two nodes of RANVIER  $N_1$  and  $N_2$  are exposed, is at the left of the bridge.  $R$ ,  $r_1$  and  $r_2$  are respectively the resistances of the order of 1 megohm, 50 ohms and 1 ohm.  $C$  is the variable condenser (below 300 pF) and  $W$  the variable resistance used to control the strength of D.C. from the battery  $B$  of about 20 volts. The untuned resistance-capacity coupled amplifier and the tuned impedance-capacity coupled amplifier are represented by RC-amp and LC-amp.  $K$ 's are the knock-over keys of a HELMHOLTZ pendulum and  $I$  is the induction coil.

The bridge output was first connected to a 2-stage resistance-capacity coupled (RC-) amplifier and then to another RC-amplifier or to a transformer coupled (LC-) amplifier tuned to the frequency of the oscillator. In the experiments of Figs 5 and 6, the output of the tuned LC-amplifier was connected to one of the deflection plates of the oscillograph and the output of the untuned RC-amplifier to the other deflection plate. In this case the A.C. component in the bridge output is superposed on the action current of the fibre. In these arrangements, the condenser  $C$ , which had a capacity of the order of  $10^{-10}$  farad, is short-circuited with a resistance of  $2.5 \cdot 10^5$  ohms in the middle of the bridge; and consequently the presence of the condenser  $C$  brings about no appreciable deformation in the action current recorded.

It should be noticed in our records that the response of the LC-amplifier to an instantaneous alteration of bridge balance was relatively slow: it took about 0.5 msec or more to complete the response. Moreover, a great instantaneous variation in the potential difference between the electrodes, such as caused by a pronounced shock artefact, could result in a damping oscillation of a similar duration in the tuning circuit of the amplifier. But, since most of the experiments described in this paper were conducted at extremely low temperatures and the duration of the action current was correspondingly long, we could obtain reliable information concerning the time course of the impedance change accompanied by the production of the action current.

## RESULTS

### 1. The impedance of the resting nerve fibre and its variation during activity

The fact that under these experimental conditions the resting impedance of the single nerve fibre is constantly well over 10 megohms has made all the high frequency



impedance measurements difficult. Frequencies over 10 kc were not used, because at such frequencies the capacity of the electrodes and the wires connected with them were considered to cause an appreciable error in the reading.

After the single fibre preparation was placed in the cooled nerve chamber and the temperature of the chamber became steady, the resting parallel resistance and capacity were measured at several frequencies between 2 and 6 kc. The resting resistance of the fibre, which could be obtained from the resistance of the known arms of the balanced bridge by multiplying the value of  $R$  in Fig. 1 by the ratio  $r_1/r_2$ , was in general between 20 and 60 megohms. It seemed fairly certain that this value varied according to the diameter and the internodal distance of the fibre. The value of  $Cr_2/r_1$  in the balanced state was approximately  $10^{-12}$  farad at a bridge frequency of 3 kc.

When the bridge was balanced with the LC-amplifier at a bridge frequency of about 3 kc, each sweep of the electron beam gave a narrow trace on the face of the oscillograph. A weak induction shock applied to the nerve fibre during the course of the sweep gave rise to a brief oscillation of the electron beam, due to the shock artefact. When the threshold was reached, the bridge went off balance and the oscillograph line was broadened into a band. Then, as the fibre recovered, the band soon narrowed down to the resting line again. In all cases the width of the band was found to vary directly as the strength of the bridge A.C. The balance was very stable and the measurement reproducible.

After the impedance change was recorded photographically as shown in Fig. 2, top left, the oscillograph was switched from the LC-amplifier to the RC-amplifier (Fig. 1) and the action current of the fibre was recorded without change of the sweep circuit of the stimulus. As the bridge oscillator was not turned off in that case, the change in the impedance resulted in a slight broadening of the oscillograph line in the action current records obtained (Fig. 2, top right).

It is easy to demonstrate the impedance change during activity at the site of stimulation. With the experimental arrangements illustrated in Fig. 1, rectangular current pulses of varying strengths could be applied to the nerve fibre through the bridge electrodes. When the bridge was balanced with A.C., a rectangular current pulse applied to the same bridge electrodes brought about a deflection of rectangular configuration on the face of the oscillograph (connected to the RC-amplifier), due to overcompensation of the stimulating current by the bridge. After the rheobase was attained, action currents of all-or-none character and simultaneous changes in the impedance were observed (Fig. 2).

As the strength of the rectangular current pulse is increased, the time interval from the onset of stimulus to the start of action current decreases, and the form of the action current becomes correspondingly diphasic as a result of the increased internodal conduction time. At a strength sufficient to suspend conduction of impulse from one node of RANVIER to the other (between  $N_1$  and  $N_2$  in Fig. 1), the action current becomes completely monophasic. At this strength, the magnitude of the impedance change was found to fall to half the normal value.

More direct information on the magnitude of impedance change during activity can be obtained, according to the technique used by COLE AND CURTIS (1941) in their experiment on the squid giant axon, by the following procedure. The resistance and capacity of the known arm of the bridge, is so altered that the bridge is no longer balanced at rest, but it becomes balanced at some definite moment during the activity (see Fig. 3). In this way, the parallel resistance of the fibre ( $Rr_1/r_2$ ) was shown to decrease by 5 to 15 % at the onset of the activity (at a bridge frequency of about 3 kc). Then, as the fibre recovers, the resistance returns to normal at a nearly constant rate until the band on the face of the oscillograph regains the resting width. The parallel capacity of the fibre ( $Cr_2/r_1$ ) was found not to alter appreciably during activity.

The magnitude of the impedance loss during activity varies pronouncedly according

to the bridge frequency. At higher frequencies the change was decidedly less. For frequencies above 7 kc it was not feasible to demonstrate any impedance change during activity.

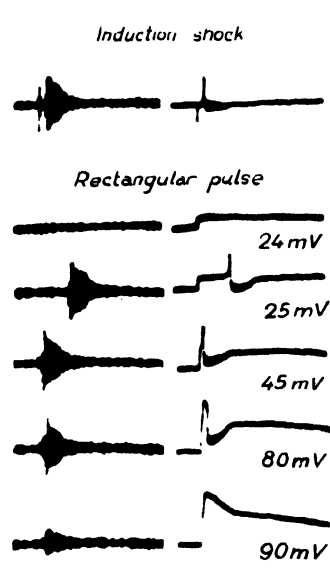


Fig. 2

Fig. 2. Left column: The impedance change during activity elicited by an induction shock (top) and rectangular current pulses (below). The bridge was balanced for the impedance of the nerve fibre at rest. The bridge frequency was 3 kc. The strength of the pulses are given in the figures. Right column: The bridge output amplified with untuned RC-amplifiers and recorded under otherwise the same condition as in the left column. The time marks at the bottom are 10 milliseconds apart. The temperature of the nerve chamber was  $3.5^{\circ}\text{C}$ .

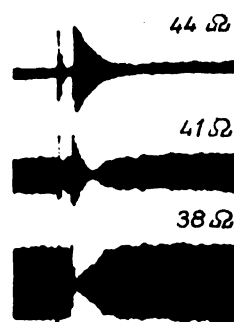


Fig. 3. Bridge output during the passage of an impulse, with the bridge balanced at rest or at various moments during action. The numerals above indicate the parallel resistances in megohms of the fibre at the balanced moments. The two lower records were obtained after application of cocaine RINGER solutions of the concentrations indicated in the figures; the resting resistance of the fibre was increased by narcosis to about 48 megohms. Frequency 2 kc; temperature  $5^{\circ}\text{C}$ ; time marks 10 msec apart.

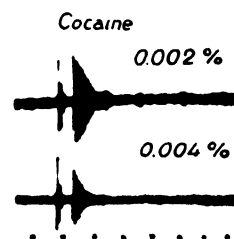


Fig. 3

## 2. The effects of alkaloids upon the time course of impedance change

Our previous investigation on the effect of alkaloids upon the isolated nerve fibre revealed that these drugs may be classified according to their physiological effects into three categories. Cocaine and many other alkaloids reduce the size and duration of the electric response of the fibre and raise the threshold; they may be referred to as narcotics. Sinomenine, brucine, emetine and heroine prolong the descending phase of the action current at adequate concentrations. Veratrine causes the fibre to produce a prolonged weak "after-current" which is the current counterpart of the negative after-potential. The classification of all alkaloids into these three types, namely into the cocaine-type, sinomenine-type and veratrine-type, become somewhat dubious at strong concentrations, as all chemicals are then narcotic in action.

In the present investigation, we have examined the effect of these three kinds of alkaloids upon the time course of the impedance change during activity. In the first place, the effect of cocaine, which is known to reduce the size and duration of the action current, was examined (Fig. 3). It was immediately disclosed that cocaine decreases the magnitude and duration of the impedance change during an action when applied to the region of the fibre around the impedance electrodes. At such strong concentrations as to inhibit completely the production of the action current, no impedance change was observed even at the site of stimulation.

We have investigated in the next place the effect of sinomenine. The drug was dissolved in RINGER at an adequate concentration (from 1 to 3%), and this solution replaced the fluid on both sides of the bridge-insulator. The fibre was excited from

time to time by means of the induction coil. Alkaloids of this type are, unlike cocaine, progressive in action, *i.e.*, the magnitude of the effect increases gradually as time elapses even when the concentration of the drug remains unaltered.

It was demonstrated that this drug remarkably prolongs the period of decreased impedance. The magnitude of the impedance change on action is much less in the poisoned fibre than in the normal, but its effect upon the time course of the change is characteristic (Fig. 4). The course is such that the oscillograph line is broadened suddenly, then it soon narrows down to a certain extent and this level is maintained for some time until it finally begins to return gradually to normal.

The effect of veratrine upon the time course of impedance change during activity is very interesting in connection with its effect on the action current. It is possible to show the impedance counterpart of the increased negative after-potential, which a number of recent investigators have demonstrated for the nerve trunk (GRAHAM, 1930; GRAHAM AND GASSER, 1931; ACHESON AND ROSENBLUTH, 1941; KUFFLER, 1943 and others).

When the RINGER's fluid on both sides of the bridge-insulator (Fig. 1) is replaced with a veratrine-RINGER solution of an appropriate concentration which varies considerably with the temperature, return of the impedance to normal following an action becomes incomplete and the band remains slightly broadened for a period of one minute or more after it has been once broadened. As the detection of the impedance change can be done fairly accurately even when the change occurs very

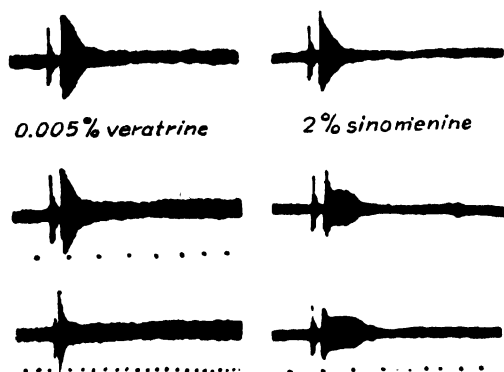


Fig. 4. Left column: The effect of veratrine as shown by photographic records of the impedance change during activity. The two lower records were obtained between 15 and 20 minutes after application of veratrine. The resting resistance was increased by the drug from 48 to 55 megohms. Frequency, 2 kc; temperature 5° C; time marks 10 msec apart. Right column: The effect of sinomenine. The two lower records were obtained about 5 and 15 minutes after application of the drug. The resting resistance was at first 50 megohms and was not much altered by the drug. Frequency 5 kc; temperature 6° C; time marks 10 msec apart.

slowly, it is obvious that, for the study of the process underlying the after-potential, the use of the impedance method is more adequate than the usual methods.

### 3. The relationship between the impedance and the action current and the effect of polarization

In order to examine the relationship between the magnitude of impedance change and the action current, a special technique was adopted, in which both the A.C. indicating the impedance changes and the action current were led simultaneously into one and the same oscillograph. In the experimental arrangement shown in Fig. 1, these two components in the bridge output were amplified first by means of the RC-amplifier (1) and then the untuned RC-amplifier (2) made it possible to record action currents of the order of  $10^{-9}$  ampere. The LC-amplifier, tuned to the bridge-A.C., had a current sensitivity of about 10 times as high as the RC-amplifier (2).

After a 0.2% cocaine-RINGER solution had been introduced into the distal pool of RINGER in which was immersed node of RANVIER  $N_2$  in the figure, an induction shock

applied to the fibre evoked an action current which was derived mainly from the activity at the node  $N_1$ . The time course of such an action current roughly duplicates that of the variation in the electromotive force at the plasma membrane of the node, namely the time course of the "action-e.m.f."

The action current records furnished in the left column of Fig. 5 were obtained with the arrangement of Fig. 1 in which the LC-amplifier was put out of action. Those in the middle column were obtained with the RC-amplifier (2) switched off. With all the amplifiers at work, the records in the right column were obtained.

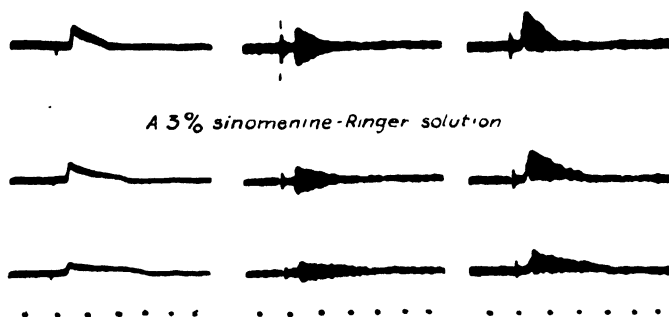


Fig. 5. Left column: "mononodal" action currents of a single nerve fibre. Middle column: Impedance changes during activity. Right column: Superposition of the impedance changes upon the action currents. Records in the two lower rows were obtained about 5 and 10 minutes after application of sinomenine respectively. Frequency 3 kc; temperature  $3^{\circ}\text{C}$ ; time markers 10 msec apart.

When the sensitivity of the RC-amplifier (2) was reduced to such an extent that the maximum width of the band was approximately equal to the maximum deflection by the action current, superposition of these two components gave figures of which the lower margins showed practically no departure from the base line of the oscillograph. And this was true even after the node under observation ( $N_1$ ) was poisoned with alkaloids (Fig. 5). This indicates undoubtedly that, if both the action current and the impedance change are limited to those derived mainly from the activity of a single node of RANVIER, *the magnitude of the impedance change is proportional to the strength of the action current at that moment.*

We came across an apparent exception to this rule in our investigation of the effect of polarization on the magnitude of impedance change during activity (Fig. 6). In this investigation, the battery circuit shown in Fig. 1 was used for the polarization of the fibre. The portion of the fibre in the distal pool

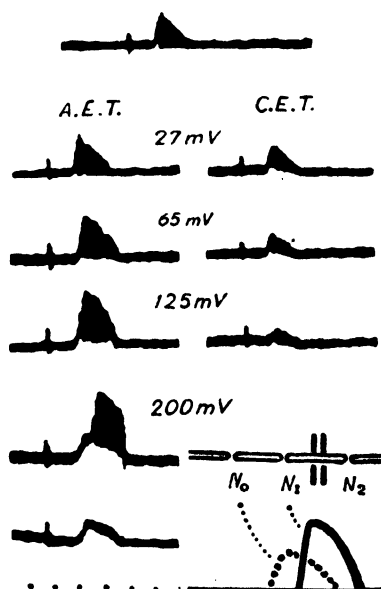


Fig. 6. Effect of electrotonic polarization upon the action current and the impedance change. Node  $N_2$  in the figure was narcotized. The records in the left column were obtained during the anodal polarization of the node  $N_1$  at various strengths, and those in the right column during cathodal polarization. Polarizing voltages are given in the figure. Two records at the bottom were taken at the same polarizing voltage. Frequency 2.8 kc, temperature  $2.5^{\circ}\text{C}$ , time marker 7 msec apart.

References p. 493.

was prevented from developing action currents by narcosis. The fibre was brought into action by an induction shock applied to its proximal portion after the polarizing current had been allowed to act for a period of about 0.5 second. In this experiment, the effect of the polarizing current is referred to as catelectrotonic (C.E.T.) when the node of RANVIER under observation ( $N_1$  in Fig. 1) is on the side of the cathode of battery B in the polarizing circuit. When the sign of the battery is reversed, we speak of anelectrotonus (A.E.T.).

By C.E.T. these two types of electric responses are reduced in size, and by A.E.T. both are increased. In the case of C.E.T., the reduction in the magnitude of the impedance change was found to be more pronounced than that in the action current, but still a proportionality seemed to exist between these two types of response in that condition. In the case of very strong A.E.T., action currents without simultaneous change are often observed. A closer analysis of this phenomenon reveals, however, that this observed disparity between the action current and the impedance change is merely superficial.

As was first discovered by ERLANGER AND BLAIR (1934), the transmission of an impulse from one node to the next is strongly hampered by A.E.T. and consequently a notch is formed on the ascending limb of the action current record. In the diagram of Fig. 6, the broken line indicates the component of the action current derived from the node  $N_0$  in the figure, and the thick continuous line shows the current from the node  $N_1$ . At the moment when the latter node  $N_1$  is thrown into action, the oscillograph line changes over suddenly from the broken line to the continuous, producing a notch in the record (TAKEUCHI AND TASAKI, 1942).

With these points in mind, it is inferred that, under these experimental conditions, the component of the current arising from the activity of the node  $N_0$  is unaccompanied by any observable change in the impedance. The observed impedance change is synchronous with and proportional to the deflection resulting from the action current of the node  $N_1$ .

#### DISCUSSION

One of the difficulties of our present investigation lies in the fact that, in order to demonstrate the change in the impedance during activity, only a restricted range of bridge frequency, 2-5 kc, is available. The limited duration of the action current determines the lower limit of the available frequency. The upper limit is influenced by the experimental fact that for higher frequencies no change in the impedance is observed during activity. This latter can no doubt be attributed to the properties of the myelin sheath which covers the nerve fibre.

There is evidence that the resistance of the myelin sheath is reactive in nature (TASAKI AND TAKEUCHI, 1942). For a direct current the sheath may behave like an almost perfect insulator, but, for a high frequency alternating current, it may act as though it were a good conductor. Adopting the view that the action current of the fibre arises from a process localized at the nodes of RANVIER, we may conclude that the inefficacy of a high frequency A.C. for detecting the impedance loss during activity is due to the leakage of the testing current through the myelin sheath.

In connection with our investigation of the effect of anodal polarization upon the impedance changes during activity, we have seen that the activity at the second node

( $N_0$ ) from the bridge-insulator may cause a sizable action current in the bridge output without being accompanied by any detectable change in the impedance. We may now attribute this too to the above-stated property of the myelin sheath. The bridge A.C. cannot reach this remote node on account of leakage through the myelin sheath, while the action current from this node may spread to the bridge electrodes after suffering an appreciable distortion in its sharp rising phase.

Turning now to the relationship between the impedance change and the action-e.m.f. at the node of RANVIER, it should first be emphasized that these two occur not only simultaneously but also their magnitudes are proportional to each other at every moment during activity. The constant of proportionality may vary according to the experimental conditions, but during one specified activity, one varies proportionately to the other. This fact indicates undoubtedly that the change in the impedance and the production of the action current are two different expressions of one and the same bioelectric process which occurs at the plasma membrane. In the experiment of COLE AND CURTIS, the measurements were made under such conditions that the diphasic artefact and the spread of the action current along the fibre could cause a slight disparity between the time courses of these two processes.

In this connection, emphasis should be laid on the peculiarity of the time course of this bioelectric process. The first, rising phase is extremely short as compared with its second, descending phase. In this respect, the bioelectric responses we recorded are very like those obtained with the plant cell *Nitella* (see for example OSTERHOUT AND HILL, 1936, p. 46).

It would be very interesting to know how this bioelectric process is affected by various chemical and physical agents such as those used in the present investigation. It is first necessary to show that the changes in the bioelectric response described above are the real effects of those agents. Their effects upon the resistance of the myelin sheath must be excluded before we can discuss the process at the node.

By the shock test method in combination with a bridge-insulator (TASAKI, 1940), it has been demonstrated that, by cocaine of above 0.2% and urethane of above 2%, the resistance of the myelin sheath is increased beyond the limit of the experimental error. In the present investigation, introduction of a 2% cocaine solution into one of the pools on the bridge-insulator increased the parallel resistance by about 5%.

With a view to clarifying the effect of the polarizing current upon the resting resistance of the nerve fibre, we have made a special experiment in which a three-electrode arrangement with the bridge-insulator technique was used. Of the three electrodes two served as the bridge-electrodes for the impedance measurement, and the remaining one in combination with one of the bridge-electrodes was used for polarizing the fibre. In the middle pool of RINGER in which the common electrode was immersed, a short portion of the fibre was introduced including one node of RANVIER. In this manner it has been shown that A.E.T. of about 100 millivolts brings about no detectable change in the resting impedance and that a slight, but unmistakable change results in case of C.E.T. of about 100 millivolts. This catelectrotonic effect is considered indistinguishable from the impedance counterpart of the after-potential which is known to increase in case of C.E.T. (GRAHAM, 1942).

Thus, all the effects of the chemical and physical agents under investigation upon the resting impedance are too small to account for the observed impedance change during activity. We may therefore conclude that all the remarkable effects brought

about by alkaloids and polarization are due to their direct action upon the action current producing process at the plasma membrane.

Among the data presented, the fact that A.E.T. augments the magnitude of the impedance changes during activity may be of considerable interest in regard to the mechanism of action current production. This seems to indicate that, contrary to the conclusion at which COLE AND CURTIS have arrived for the squid giant axon, the decrease in the membrane resistance during activity is not so profound as to be regarded as a complete depolarization of the membrane. If the resistance of the normal plasma membrane is decreased on action by almost 100% it would be impossible for the impedance during activity to be augmented by any kind of agent which brought about no change in the resting resistance.

Some of our previous results, however, strongly suggest that the ohmic resistance of an active plasma membrane would be practically zero compared with its resting resistance (TASAKI, 1940; TASAKI AND TAKEUCHI, 1942). Neither stimulating nor action current spreads beyond an active node of RANVIER. But, since the accuracy of this method of measuring the membrane resistance falls far below the direct impedance measurement, it would probably be possible to find a way to reconcile these two sets of experimental results without introducing any complicated assumptions.

We wish to express to Dr WASABAYASHI AND Dr KATSUKI our appreciation of their kindness in lending us some experimental equipment; and to Dr OCHIAI AND Dr MIYAGI our thanks for supplying us with alkaloids.

#### SUMMARY

1. High frequency alternating current impedance measurements have been made, during rest and activity, on isolated single nerve fibres of the toad.
2. During activity there is a decrease in the impedance of the plasma membrane at the nodes of RANVIER. The time course of the impedance loss is the same as that of the "action electromotive force" set up at the plasma membrane.
3. The impedance change and the action current production are two different expressions of one and the same bioelectric process which occurs at the plasma membrane.
4. Cocaine and many other alkaloids reduce the magnitude and duration of the bioelectric response. Sinomenine and several other alkaloids prolong the duration of the response in a very characteristic manner. Veratrine retards complete recovery from a previous activity.
5. Catelectrotonus depresses and anelectrotonus enhances the bioelectric process.

#### RÉSUMÉ

1. Des mesures d'impédance ont été faites sur des fibres nerveuses isolées, de crapaud, au cours du repos et de l'activité, à l'aide de courant de haute fréquence.
2. Au cours de l'activité, il y a une diminution de l'impédance de la membrane protoplasmique aux noeuds de RANVIER. La diminution de l'impédance en fonction du temps est la même que celle de la "force d'action électromotrice" qui se manifeste à la membrane protoplasmique.
3. Le changement d'impédance et la production du courant d'action représentent deux manifestations différentes d'un seul et même phénomène bioélectrique qui a lieu à la membrane protoplasmique.
4. La cocaïne et de nombreux autres alcaloïdes réduisent la grandeur et la durée de la réponse bioélectrique. La sinoménine et plusieurs autres alcaloïdes prolongent la durée de la réponse d'une façon très caractéristique. La veratrine ralentit le retour à la normale après activité.
5. Le catélectrotonus diminue et l'anélectrotonus augmente le phénomène bioélectrique.

## ZUSAMMENFASSUNG

1. Die Impedanz von Hochfrequenz-Wechselströmen sowohl im Ruhe- als im Arbeitszustand, wurde an isolierten Nervensträngen der Kröte gemessen.
2. Während der Arbeit findet eine Abnahme der Impedanz der Plasma-Membran bei den RANVIER-Knoten statt. Der Zeitverlauf dieses Impedanz-Verlustes ist der gleiche als der der "elektromotorischen Wirkungskraft" welche an der Plasma-Membran entsteht.
3. Die Impedanz-Änderung und die Erzeugung des Wirkungsstromes sind zwei verschiedene Ausdrücke eines und desselben bioelektrischen Prozesses, welcher an der Plasma-Membran stattfindet.
4. Cocain und viele andere Alkaloide verringern die Grösse und Dauer der bioelektrischen Ansprechnung. Sinomenin und verschiedene weitere Alkaloide verlängern die Dauer der Ansprechnung in sehr charakteristischer Weise. Veratrin verzögert die völlige Erholung von vorgängiger Arbeit.
5. Katelektrotonus senkt und Anelektrotonus erhöht den bioelektrischen Prozess.

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## COLLISION OF TWO NERVE IMPULSES IN THE NERVE FIBRE

by

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A previous paper (TASAKI AND TAKEUCHI, 1942) indicated that it is possible to lead the action current of a nerve fibre through the myelin sheath. The method consisted in dividing the fluid which surrounds the fibre, by means of two sets of bridge-insulators placed between two neighbouring nodes of RANVIER (see Fig. 1, top), into three independent pools. The electrode dipping in the middle pool of RINGER was led to the grid of the amplifier and those immersed in the two lateral pools were earthed. The fibre was brought into action by means of induction shocks applied to the portion near its proximal or distal stump.

The action current of a single nerve fibre recorded in this manner is of very short duration and has two peaks in its course (Fig. 1, a and b). The time interval between these two peaks is, at room temperature, about 0.1 millisecond, which corresponds to the time required for conduction of the impulse from one node of RANVIER to the next. If the fluid on the distal side (with respect to the stimulating electrodes) of the bridge-insulators is replaced by a narcotizing solution, the second peak alone is extinguished. Introduction of the same narcotizing solution into the middle pool brings about no detectable change in the action current observed.

This method of leading off the action current of a nerve fibre provides us with a very simple means of observing the collision of two nerve impulses at the site of the lead-off electrodes. The purpose of this paper is to present some data obtained by this and another method showing what happens when two impulses, which are made to start at both ends of a nerve fibre, come to the same point from two opposite directions. An outline of this work was included in my Japanese monograph, *Physiology of the nerve fibre*, published in 1944.

## METHOD

Motor nerve fibres innervating the sartorius muscle of the toad were used for the experiments. The operation of isolating a single nerve fibre was carried out at a point on the nerve about 25 mm from the muscle. In the first experiment, the width of the middle pool of RINGER was about 0.5 mm (Fig. 1, top), and the gaps between the pools which served to insulate the three pools from one another were about 0.2 mm wide. As the internodal distance of a "rapid" motor nerve fibre of the toad is in favourable cases well over 2 mm, it is easy to mount the fibre in such a manner that the two exposed nodes of RANVIER are situated in the two lateral pools and only the myelinated portion of the fibre is dipped in the middle pool.

In the second experiment, a node of RANVIER was introduced into the middle pool of RINGER as shown in Fig. 2, top. The middle pool was in this case 0.5–1.5 mm wide, and the gaps were about 0.2 mm wide as usual. The non-polarizable electrode dipped in the middle pool was led to the amplifier and those immersed in the two lateral pools were earthed as in the first experiment.

The portions of the nerve on both sides of the operative region were suspended in the air and

were brought in contact with two pairs of platinum electrodes. Each pair of the platinum electrodes was connected to the secondary coil of an inductorium. The strength of the induction shock was controlled by means of precision resistances in the primary circuits. Break contacts of a HELMHOLTZ pendulum inserted in the primary circuits of these two induction coils served to control the time interval between the two shocks.

The action currents were recorded with a cathode ray oscillograph of which a single sweep was started by the use of another knock-over key of the pendulum. The input resistance of the amplifier was about 0.1 megohm.

### RESULTS

In the experimental arrangement shown in Fig. 1, top, an induction shock applied to one end of the nerve fibre set up an action current of the double-peaked configuration (record a in the figure). A stimulus applied to the other end of the fibre caused a similar action current (record b), but the conduction time was in general different, due to a different conduction distance. The time interval from the start of the sweep of the cathode ray to the onset of each of the induction shocks was so adjusted that the figures of the action currents appeared at exactly the same spot on the face of the oscillograph in both cases. Then, without change in the position of all the three break contacts of the pendulum, two induction shocks were applied to the

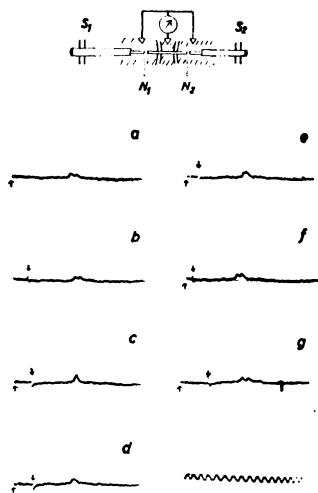


Fig. 1. Collision of two nerve impulses as observed with an amplifier and a cathode ray oscillograph connected to the myelin covered portion of a single nerve fibre. In a, induction shock  $S_1$  alone was delivered. The arrow indicates the position of the shock artefact. In b, the fibre was excited by shock  $S_2$ . In c, both  $S_1$  and  $S_2$  were applied to the fibre in such a manner that the two impulses arrived at the region of the lead-off electrodes simultaneously. In d and g, the impulse from  $S_1$  preceded that from  $S_2$  by about 0.10 and 0.32 millisecond respectively. In e and f, the impulse from  $S_1$  lagged behind that from  $S_2$  by approximately 0.10 and 0.22 millisecond respectively. A.C. at the bottom 5000 cycles per second. Temperature  $21^\circ\text{C}$ .

fibre in succession. By this procedure, the two impulses starting at the two opposite ends of the nerve fibre were made to collide at the region of the bridge-insulators, and this yielded, as may be expected a single-peaked action current record (c in the figure).

A point of interest in this experiment is that a singlepeaked record is obtainable even when two impulses do not reach the middle pool at exactly the same moment. When one impulse lags behind the other by about 0.1 millisecond which corresponds to the internodal conduction time, the peak is much lower than when the two arrive simultaneously, but still the impulses are found to fuse to form a single-peaked action current. When one of the impulses lags behind the other by a still greater period, ordinary records of the double-peaked configuration are obtained. Introduction of a 0.2% cocaine RINGER solution into the middle pool brings about practically no change in the configuration of these action currents.

It is obvious in this experiment that the first peak in the action current record corresponds to the initiation of activity at the proximal node ( $N_1$  in the case when the fibre is excited by  $S_1$ ) and the second peak to that at the distal node. The variation in the electromotive force at the plasma membrane of the node, namely the "action-e.m.f.", reaches a maximum in a period much shorter than 0.1 millisecond at room temperature

and then decays almost linearly with time until finally the electromotive force returns to normal about 1 millisecond after the onset of the variation. This action-e.m.f. causes, through the myelin sheath of the fibre, a current which lasts for only about 0.2 millisecond. The myelin sheath is composed of a highly polarizable membrane and the portion of the fibre in the middle pool behaves like a small condenser.

In the experiments in which a node of RANVIER is introduced into the middle pool of RINGER (Fig. 2), the action current recorded is always of a diphasic configuration (record a). When the nerve impulse set up by the induction shock  $S_1$  reaches the node  $N_0$  in the figure, the node  $N_1$  is traversed by a strong outward-directed current. At the moment when this latter node is also thrown into action as the result of stimulation by the outward-directed current, the current through the axis-cylinder between the node  $N_0$  and  $N_1$  ceases at once, for the action-e.m.f. at the latter node tends to cause a current in the opposite direction. But at this moment a strong outward-directed current begins to flow through the node  $N_2$ , owing to the start of action-e.m.f. at the middle node  $N_1$  (TASAKI, 1939, Fig. 12). This in turn results in an inward-directed current through the middle node  $N_1$ , producing a downward deflection in the action current record.

When the distal node  $N_2$  is finally brought into action, the current through the axis-cylinder between  $N_1$  and  $N_2$  is also terminated, and the current through the middle node  $N_1$  becomes almost zero. In these considerations, the leakage of

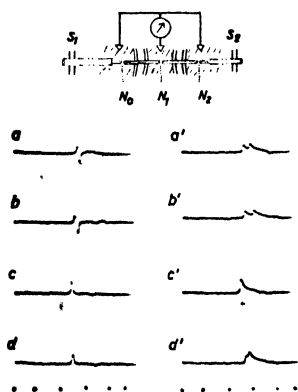


Fig. 2. Records of the currents which traverse a node of RANVIER when an impulse travels along a nerve fibre (a, b, a' and b') and when two impulses collide at the node in the middle pool (c and c'). Records in the right column were obtained after introduction of a 0.3 % cocaine RINGER solution into the middle pool where the node  $N_1$  lay. In d and d', the impulse from  $S_1$  lagged behind that from  $S_2$  by 0.05 and 0.2 millisecond respectively. The time markers 1 millisecond apart. Temp. 21° C.

current through the myelin sheath is disregarded as being too small compared with the current through the node.

Let us now consider what happens when two impulses collide at the middle node. When the node  $N_0$  and  $N_2$  are brought into action simultaneously, the node  $N_1$  in between begins to be traversed by an outward-directed current much stronger than that in the ordinary transmission. This strong outward-directed current naturally excites the node  $N_1$  very readily, and the latency in excitation is correspondingly shorter than 0.1 millisecond. In other words, when two impulses approach one another, they travel from node to node at a rate much greater than in the ordinary transmission. When the node  $N_1$  is finally brought into action, the strength of the current flowing in- and outside the fibre becomes practically zero. It should be emphasized that, even though there is practically no current along the fibre at this stage, the activity at these nodes is still under way. This activity subsides of itself, by some mechanism inherent to the bioelectric process, in about 1 millisecond from the onset.

In the records of Fig. 2, left, one may notice slight movements of the base line after the prominent deflections have ended. This instability of the base line lasts for about 1 millisecond and is evidently due to slight differences in the magnitudes of the action-e.m.f. at the nodes under investigation.

Some physiologists seem to believe that, when two impulses collide at one spot on a nerve fibre, the transmission of each of these impulses is blocked by refractoriness left behind by the other impulse. But this is an utterly mistaken idea. We have seen above that, at the moment when the last node of RANVIER in the fibre is involved in action, the current in- and outside the fiber becomes insignificantly small. In other words, there is in this stage no internal stimulus through which the transmission is effected. It is a property of the bioelectric process to subside by itself.

Records in the right column of Fig. 2 were obtained after introduction of a 0.3% cocaine RINGER solution into the middle pool in which the node  $N_1$  was immersed. When the impulse starting at  $S_1$  in the figure reaches the node  $N_0$ , the middle node is traversed by an outward-directed current. The first peak in the record a' of Fig. 2 corresponds to the beginning of activity at the node  $N_0$ . As the middle node  $N_1$  fails to respond to this outward-directed current, there occurs spread of current along the fibre; and after a latent period much longer than the ordinary internodal conduction time, the distal node  $N_2$  is brought into action by this spreading current (TASAKI, 1939). The second peak in the record a' corresponds to the onset of the action-e.m.f. at the node  $N_2$ . In these circumstances, collision of two impulses at this point yields such records as c' and d' in Fig. 2.

#### SUMMARY

1. Records have been taken of the current that traverses the myelin sheath and the node of RANVIER as an impulse travels along a nerve fibre.
2. Records of action currents have been obtained from the spot on a nerve fibre at which collision of two impulses has occurred.
3. When two impulses approach one another, the rate of transmission becomes greater.
4. By collision, transmission of impulses is blocked, not on account of the refractoriness left behind by the impulses, but through lack of internal stimulating current by which the normal transmission is effected.

#### RÉSUMÉ

1. Des enregistrements ont été pris du courant traversant la gaine de myéline et le nœud de RANVIER lorsqu'un influx se propage le long d'une fibre nerveuse.
2. On a obtenu des enregistrements de courants d'action provenant du lieu de rencontre de deux influx dans une fibre nerveuse.
3. Lorsque deux influx se rapprochent l'un de l'autre, la vitesse de leur transmission s'accroît.
4. Lorsqu'il y a collision, la transmission des influx est bloquée, non pas par suite de la période réfractaire qui suit ces influx, mais à cause de l'absence d'un courant d'excitation interne par l'intermédiaire duquel la transmission normale est effectuée.

#### ZUSAMMENFASSUNG

1. Es sind Aufnahmen gemacht worden von dem Strom welcher die Myelinscheide und den RANVIER-Knoten durchläuft, wenn eine Anregung sich entlang einer Nervenfiber bewegt.
2. Aufnahmen von Wirkungsströmen sind erhalten worden von der Stelle wo der Zusammenstoß von zwei Anregungen stattgefunden hat.
3. Wenn zwei Anregungen sich einander nähern, wächst ihre Fortpflanzungsgeschwindigkeit.
4. Beim Zusammenstoß wird die Fortpflanzung der Anregungen gesperrt, nicht wegen der Unempfindlichkeit welche diese Anregungen zurücklassen, sondern mangels eines internen Erregungsstromes, durch welchen die normale Fortpflanzung bewirkt wird.

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## THE EXCITATORY AND RECOVERY PROCESSES IN THE NERVE FIBRE AS MODIFIED BY TEMPERATURE CHANGES

by

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In a preceding paper (TASAKI AND FUJITA, 1948), the results were reported of our investigation of the effect of temperature changes on the action current of a single myelinated nerve fibre. In the present paper, it is proposed to present data on the effect of temperature on the excitatory process set up by electric stimuli and upon the process of recovery from a previous activity.

The excitability characteristics of the nerve fibre examined in this investigation are (1) the rheobase, (2) the minimal gradient or the constant of accommodation, (3) the minimum quantity of electricity required for excitation, (4) the time course of development of the excitatory state set up by a brief subthreshold shock, and (5) the recovery curve.

The effects of temperature on these quantities are a much discussed subject, and the present investigation is in a sense a re-examination, with isolated single nerve fibres, of previous experiments carried out by GOTCH AND McDONALD (1896), SCHRIEVER (1932), ADRIAN (1914) and many others. But, since it has been revealed that the rules governing the process of electric excitation of an isolated single nerve fibre are somewhat different from those already known (TASAKI, 1942), it has been thought worth while to secure rigid experimental data showing how these quantities are affected by the change in temperature.

### METHOD IN GENERAL

The material used was isolated single motor nerve fibres of the toad exclusively. The method of isolation and stimulation was the same as that adopted previously (TASAKI AND FUJITA, 1948). The single fibre preparation was mounted on a bridge-insulator and was introduced, together with the stimulating and lead-off electrodes, into a special nerve chamber of which the temperature was controlled by ice or by an electric hot plate supplied with direct current.

### PROCEDURES AND RESULTS

#### 1. *The effect of temperature upon the rheobase and the minimal gradient*

In this series of experiments, the arrangement shown in Fig. 1 was used. With the contact  $K_2$  in the figure closed, opening of the contact  $K_1$  initiated an exponentially rising stimulating voltage. The time constant of voltage rise was controlled by changing the capacity of the condenser C in the figure, and the final voltage was adjusted to threshold by means of a potential divider of 1 000 ohms. The stimulating current was interrupted by opening the second contact  $K_2$  about 0.5 second after the onset of the stimulus. The pause between each trial (stimulation) was about 60 seconds. The time required for readjusting the temperature in the nerve chamber was about 10 minutes. And about 10 minutes after the temperature of the chamber had become steady at a new level, threshold deter-

*References p. 508/509.*

minations were begun. In this experiment, the response of the muscle was generally taken as index of nerve excitation.

In isolated single nerve fibres, as in the whole nerve trunk (SCHRIEVER, 1930; SOLANDT, 1936 and others), the relation between the time constant of voltage rise and the final voltage which barely excites is represented by a straight line. It should be added however that the deviation of the observed relation from a straight line is far less in the single nerve fibres than in the nerve trunks.

The data presented in Fig. 1, left, are an example of the results of the investigation. In this figure, the final voltage  $V$  is plotted as ordinate against the time constant of voltage rise  $\tau c$  as abscissa. The rheobase (LAPICQUE, 1909) is the threshold voltage for  $\tau c = 0$ , and the minimal gradient (LUCAS, 1907) is given by the slope of the straight line relating  $V$  and  $\tau c$ . HILL's time constant of accommodation (1936) is given by the rheobase into the reciprocal of LUCAS's minimal gradient.

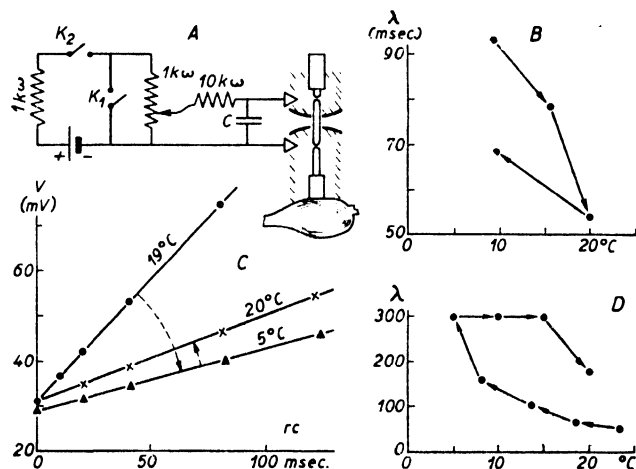


Fig. 1. A: Arrangement used to determine the threshold for exponentially rising voltages. B: Time constant of accommodation determined at four different temperatures. The arrows indicate the order of the measurement. C: The relation between the time constant of voltage rise  $\tau c$  and the threshold final voltage  $V$  in excitation by exponentially rising voltages at various temperature. D: Time constant of accommodation determined at eight different temperatures. The preparation is the same as that used in C, and three points are taken from the data of C.

According to the conclusion reached in our previous investigation (SAKAGUCHI AND TASAKI, 1943), the necessary and sufficient condition for a slowly rising voltage  $V(t)$  to excite a nerve fibre is to make the stimulating voltage cross the rheobasic voltage  $b$  at a rate of rise greater than the minimal gradient  $m$  of the fibre, *i.e.*, at the moment when  $V(t) = b$ ,  $dV(t)/dt \geq m$ . It can be easily shown that, if this requirement is fulfilled by a set of exponentially rising voltages with varying constants of rise, the relation between the constant of voltage rise  $\tau c$  and the final threshold voltage  $V$  should be expressed by a straight line which intersects the voltage axis at a slope equal to the minimal gradient  $m$ . In excitation by these slowly rising voltages, action currents are set up soon after the stimulating voltages surpass the rheobase (see Appendix I).

The experimental result furnished in Fig. 1 indicates very clearly that the minimal gradient shows a tendency to be decreased by cold while the rheobase remains practically unaffected by temperature changes. It is further demonstrated that the minimal gradient is not a simple function of the temperature but it varies according to the history of the

fibre. The data presented in Fig. 1, B and D, show how it depends upon the sequence of the measurement. In these figures the data are presented in terms of HILL's time constant.

Demonstration of hysteresis in the effect of temperature changes upon the minimal gradient of the nerve would probably not be very new, although COPPÉE (1940) did not seem to pay much attention to this phenomenon. SCHRIEVER has shown, in 1932, that his "Einschleichzeit" gradually changes when the nerve is subjected to constant, low temperature. His data clearly indicate that at a constant temperature the rheobase and the chronaxie of the nerve remain practically constant while the minimal gradient changes gradually as time elapses. He has further shown, in collaboration with CEBULLA (1938), that preparations taken from frogs which have been kept at low temperature show distinctly smaller minimal gradients than those taken from ordinary frogs.

It should be pointed out now that, among all the quantities characterizing the state of the nerve fibre so far examined, the minimal gradient is the only one that shows a pronounced hysteresis. It is in fact surprising that a nerve fibre is capable of retaining the effect of its previous environment.

Turning now to the effect of temperature changes upon the rheobase, all the previous investigations seem to indicate that it is significantly decreased by cold (GOTCH AND McDONALD, 1896; WALLER, 1899; SCHRIEVER, 1932; SUZUKI, 1939 and many others). In the experiments on single nerve fibres, there seems also a slight tendency for it to be decreased by cold, but this tendency is too small to account for the results with nerve trunk.

In the experiments in which whole nerve trunks are used, the rheobase is considered to be strongly affected by the changes in the resistance and polarizability of the surrounding tissues and the body fluid. This effect of the shunt seems to become especially significant when the rheobase is measured in terms of the current, and not in voltages (SUZUKI, 1939). Thus the discrepancy between the present and previous results may probably be accounted for by some complication in the latter case resulting from the surrounding tissues.

## 2. *The minimum quantity of electricity required for excitation and the time course of the excitatory state*

In this series of experiments, the effect of a subthreshold current pulse, either a brief shock or a long rectangular pulse, was investigated by the shock test method at varying temperatures. The arrangement shown in Fig. 2, A, was generally used. Test and conditioning shocks of about 0.03 millisecond in duration were obtained with a HELMHOLTZ pendulum by the technique described by HOZAWA (1928); P and P' in the figure indicate keys which close the circuit for such a brief period. When a long rectangular voltage pulse was to be employed as the conditioning stimulus, ordinary break contacts K<sub>1</sub> and K<sub>2</sub> of the pendulum were used instead of the "Punktkontakt" P'. The time intervals between the shocks were controlled by shifting the positions of the contacts of the pendulum. The strengths of the shocks were varied by means of potential dividers.

In the example of the experimental results furnished in Fig. 2, the direction of the conditioning shock was ascending with respect to the muscle and that of the test shock was descending. At 15° C (B in the figure), the threshold for the test shock (applied alone) was 0.34 volt. This resting threshold level was modified by a conditioning shock of 0.2 volt in strength and about 0.03 millisecond in duration as indicated by the circles connected with a continuous line. In this figure, the threshold for the test shock is plotted against the time interval from the onset of the conditioning shock to the beginning of the test shock. A constant voltage of 0.02 volt changed the resting threshold level as indicated by the triangles connected with a broken line.

When this preparation was cooled to  $3^{\circ}\text{C}$  (C in the figure), the resting threshold for the test shock increased to 0.74 volt, and this threshold level was modified by the conditioning stimuli as shown by the curves in the figure. The time course of the whole process is decidedly slower at the lower temperature than at the higher temperature.

Attention should be called in these experiments to the fact that the threshold level for the test shock is characteristically modified by the conditioning stimulus even when the test shock precedes the conditioning stimulus. An explanation of this experimental fact has been fully described in a previous paper (TASAKI, 1942). An outline of that explanation is as follows:

The excitatory state set up in the nerve fibre by a brief subthreshold shock does not reach the maximum at the moment at which the shock terminates. This state rises first gradually and then quickly, and reaches a maximum about 0.3 millisecond after the termination of the brief shock (at room temperature). After this maximum is reached it finally begins to decay. As a result of this delayed maximum, the "excitability change" as revealed by the shock test method gives us the information on the state of the fibre about 0.3 millisecond after application of the test shock.

The magnitude of the excitatory state increases proportionately with increasing shock strength. When many subthreshold shocks are applied to the nerve fibre in succession, the excitatory states set up by these individual shocks are superposed upon one another. As a consequence of this law of superposition, the excitatory state set up by a long rectangular voltage pulse is given by the integration of the effects of all the voltage elements of which the continuous voltage can be regarded as composed (see Appendix II).

In the experimental result presented in Fig. 2, one may at once notice that, at every temperature, the gradient (or the first derivative) of the broken line gives a curve which closely resembles the continuous line. This fact is in accordance with the statement outlined above. It is also to be expected from the above statement that the broken line should be obtained by computing the area between the dotted line (representing the resting threshold level) and the continuous line (from  $t = -\infty$  to an arbitrary value of  $t$ ) and multiplying the area obtained by 0.1 (the ratio of the voltages in both cases). On actual computation by the graphical method, one finds that this requirement is fulfilled to a first approximation (cf. Appendix, equation 6).

The data presented in Fig. 3, left, indicate that, at every temperature, the magnitude of the change in the threshold for the test shock is proportional to the strength of the conditioning rectangular voltage pulse. Test shocks were in this case condenser discharges of time constant 0.02 millisecond and were applied 4 milliseconds after the onset of conditioning stimuli of varying strengths. The observed values lie on good straight lines, except in cases where the conditioning stimuli are near the rheobase.

References p. 508/509.

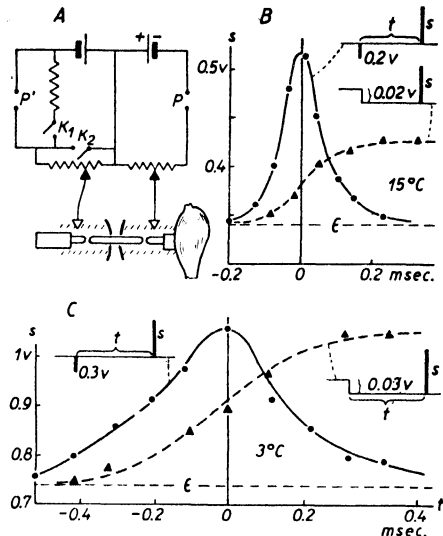


Fig. 2. A: Arrangement used to determine the variation in the threshold resulting from application of a brief subthreshold shock or a long rectangular current pulse. P's are HOZAWA's "Punktkontakt", and K's break contacts of a HELMHOLTZ pendulum. B: Curves showing the variation in the threshold caused by a brief shock and a long rectangular pulse determined at  $15^{\circ}\text{C}$ . The strengths of the conditioning stimuli are given in the figure. C: Similar experiment done at  $3^{\circ}\text{C}$  on the same preparation.



In the above conception of treating the experimental data, two quantities serve to characterize the state of the nerve fibre: the minimum quantity of electricity required for excitation and a time constant which measures the period during which the excitatory state set up by a brief shock persists. Since it is not necessary here to know the absolute value of the quantity of electricity, one may represent it simply by the product of the strength of the threshold shock into its duration. As the time constant, one may adopt the area under the "latent addition curve" (the curve indicating the threshold variation set up by a brief shock) of which the height is taken as unity.

From the above relationship between the continuous and broken lines in Fig. 2, the total area under the latent addition curve can be obtained from the plateau level of the broken line, or, what is the same thing, from the slope of the straight line obtained

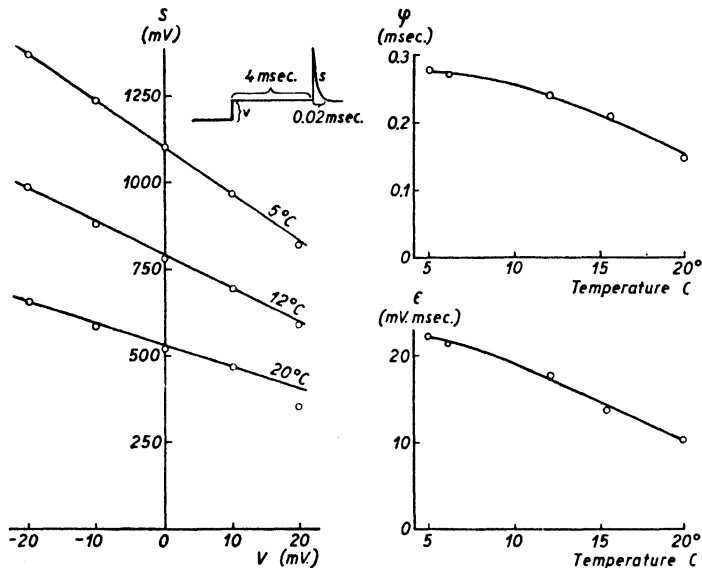


Fig. 3. Left: Relation between the strength of the (conditioning) rectangular voltage pulse  $v$  and the threshold for the (test) shock  $S$  determined at various temperatures. Right: The area under the latent addition curve,  $\phi$ , and the minimum quantity of electricity required for excitation,  $\epsilon$ , as functions of temperature. All the data in this figure are obtained for one preparation.

in the experiment of Fig. 3, left. The slopes of these straight lines multiplied by the duration of the test shock gives the area (having a dimension of time) under the latent addition curve (see Appendix, equation 7).

An example of the experimental results furnished in Fig. 3, right, shows how the minimum quantity for excitation (top) and the area under the latent addition curve (bottom) vary as the temperature. The changes in these quantities resulting from temperature changes are reversible, *i.e.*, there is no hysteresis. Both of them are increased by cold. The changes in the two quantities are parallel. Between 8 and 20° C the temperature coefficient was approximately 1.7. At low temperature there seems to be a slight decrease in the temperature coefficient, but it is possible that this is due to some error resulting from the inadequacy of the nerve chamber used in this experiment.

There is an extensive literature dealing with the effect of temperature changes

upon the excitability, the chronaxie and the strength-duration relation of the nerve (GOTCH AND McDONALD, 1896; LUCAS AND MINES, 1907; L. AND Mme LAPICQUE, 1907; GRANBERG AND HOLLANDER, 1927; DWORKIN AND FLORKIN, 1930; HOU, 1931; SCHRIEVER, 1932; BLAIR, 1935; SUZUKI, 1939, and others). In the present investigation, I made no direct measurement of the temperature coefficient for the strength-duration relation and the chronaxie. But, since WEISS' empirical formula holds good for the strength-duration relation in the single nerve fibre and consequently the chronaxie is given at every temperature by the minimum quantity divided by the rheobase, the effect of temperature upon the chronaxie is already evident from the experimental results stated above. As the rheobase is shown not to vary appreciably with temperature, the change in the minimum quantity indicates directly how the chronaxie is affected by temperature. The coefficient of about 1.7 given above seems to agree well with most of the previous results.

### 3. The recovery curve

When one sends two induction shocks into a nerve fibre through a pair of electrodes placed on the nerve trunk of a single nerve fibre preparation, one generally obtains a

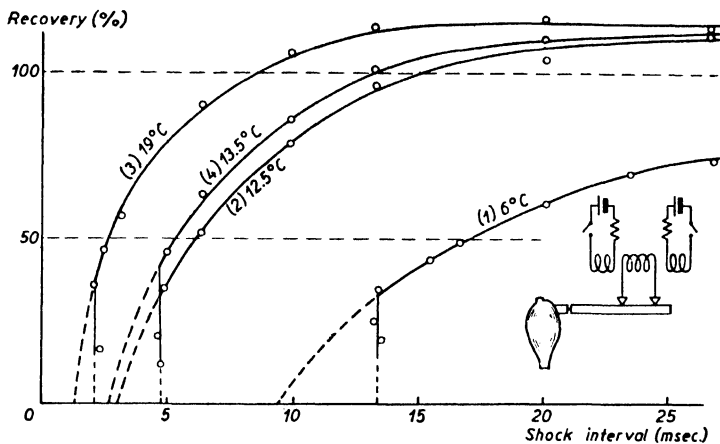


Fig. 4. The recovery curves of a nerve fibre determined at four different temperatures. The numerals in parentheses indicate the order of the determination. The broken lines are so drawn that the whole curves can be expressed roughly by continuous exponential functions.

recovery curve such as shown in Fig. 4. In this figure, the reciprocal of the threshold for the second shock, as a percentage of the resting value, is plotted against the interval between these two shocks. Cold retards the process of recovery remarkably, as was first demonstrated by BRAMWELL AND LUCAS (1911).

In the experiment of Fig. 4, fluid electrodes of the type used by ADRIAN (1914) were employed. The preparation was placed in the nerve chamber, and the recovery curve was determined by taking as index the action current of the single nerve fibre. The strength of the first induction shock was fixed at double the resting threshold.

The recovery curve of a single nerve fibre thus obtained is discontinuous; the curves always become vertical at a certain time interval. This fact has often been noticed by ADRIAN (1914), who attributed it to some kind of decreasing conduction of the second impulse. In a previous paper (TASAKI AND TAKEUCHI, 1942) direct evidence has been

presented indicating that this discontinuity is due to a decrease in the safety factor of transmission during the refractory period. In the early stage of the refractory period the threshold of individual nodes of RANVIER at the site of stimulation is so decreased that the reduced action current becomes ineffective in exciting the neighbouring node.

The discontinuity is thus a direct consequence of the electric transmission of the impulse, and the recovery curve without such discontinuity is due to some artefact resulting from the use of the propagated multifibre response as index.

Quantitative consideration leads us further to infer that the recovery of excitability at the interval where the recovery curve becomes discontinuous should be greater than about 20%. The safety factor in the resting fibre is about 5 (TASAKI AND TAKEUCHI, 1942); this means that, if the excitability of the node is decreased to 1/5th the normal value, transmission should be suspended even when the size of the electric response from the individual node remains normal. Since the size of the response, as is well known, decreases during the refractory period, it should be impossible for transmission to take place when the excitability is only 20% of normal. Although the problem of the duration of the testing current is considered to complicate the matter somewhat, all the experimental data hitherto obtained seem to verify this inference.

At the site of stimulation there is naturally no such discontinuity in the recovery curve, and the extension of the recovery curve indicated by the broken lines in Fig. 4 can actually be observed. It is therefore wise to distinguish the "absolutely refractory" period from the "non-conducting phase" in the relatively refractory period. The end of the non-conducting phase may be properly termed the "least interval" required for setting up two transmitted impulses in the nerve fibre.

Turning now to the temperature coefficient of the recovery process, the variation in the duration of the absolutely refractory period resulting from temperature changes is best discussed in connection with that in the spike duration. In the preceding paper, it was shown that the temperature coefficient of the spike duration is 3.5 for a change of  $10^{\circ}$ . If the duration of the absolutely refractory period has a temperature coefficient very different from that of the spike duration, the time interval between the end of the spike and the earliest return of excitability should vary according to the temperature.

If, on the contrary, there is no difference in the temperature coefficients of the two processes, then the relative positions of the spike and the end of the absolutely refractory period should remain unaffected by a change of temperature.

Experiments carried out to test this point indicated that there is no detectable difference in the temperature coefficients. It was shown further that, as ADRIAN (1921) has pointed out, the end of the spike always coincides with the end of the absolutely refractory period, regardless of the temperature. Fig. 5 shows the method and an example of the results of the experiments.

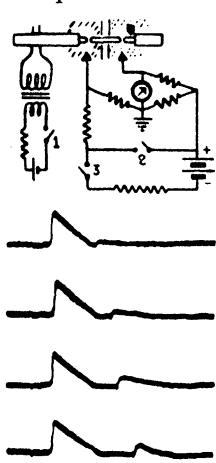


Fig. 5. Action currents of a nerve fibre evoked during the "non-conducting phase" in the relatively refractory period. The first spikes were induced by induction shocks, and the second subnormal spikes by rectangular pulses of 200 millivolts. The interval between the two stimuli was varied at a step of 1.5 millisecond. Temperature was between  $8$  and  $7^{\circ}$  C. Time marks below 1 millisecond apart.

A single motor nerve fibre, mounted on a bridge-insulator, was excited by an induction shock applied to the nerve trunk. As the portion of the fibre on the distal side of the bridge-insulator had been treated with a 0.2 % cocaine Ringer solution, the action current was completely "mononodal". The second stimulus, a rectangular current pulse, was applied to the operative region of the fibre. To suppress the displacement of the base line in the action current record by the stimulating current, the technique of the direct current Wheatstone bridge was used. The interval between the first (induction) shock and the second (rectangular) pulse was controlled with a HELMHOLTZ pendulum. The strength of the second pulse was about 6 times the rheobase.

As will be seen in the records, the size of the second response decreases continuously with decreasing shock interval. The relation between the size of the response and the interval between the stimuli indicates that the earliest return of excitability occurs immediately after the end of the spike. And this was true in all five experiments done at varying temperatures between 5 and 17° C. The interval from the end of the first spike to the earliest second spike (obtained by the extrapolation) was always less than about 15% of the total spike duration.

The effect of temperature changes on the time course of the whole recovery curve was investigated in only two cases. In the example illustrated in Fig. 4, the temperature coefficient for the 50% recovery was between 3.3 and 3.8. In the other case, in which determinations were made at 13°, 7° and 19° C, the coefficient was found to be about 4. Although the accuracy of these determinations is not very satisfactory, it seems safe to conclude that, confirming ADRIAN's previous finding, the recovery curve is affected by the temperature changes to the same extent as the spike duration.

All the findings described above are in general in good agreement with the results obtained by previous investigators. In 1914, ADRIAN found that the temperature coefficient of the recovery curve is the same whatever the specific percentage of recovery at which the comparison is made. AMBERSON (1930) made a thorough investigation on the temperature coefficient of "the least interval" for the double impulse. These authors, as well as GASSER (1931) who made use of a cathode ray oscillograph for the experiment, obtained a coefficient of approximately 3. In the results obtained by SCHOEPLF AND ERLANGER (1941) on single nerve fibres, the coefficient varied as the temperature; at lower temperature, it seemed to approach about 4.

#### DISCUSSION

In a preceding paper, it was shown that, in single nerve fibres of the toad, the temperature coefficient for the conduction rate, the spike height and the spike duration are 1.8, 1.3 and 3.5, respectively. By cold, the process of conduction and of recovery are retarded and the spike height is slightly decreased. After the manuscript of our paper had been submitted for publication in this journal, the reprint of SCHOEPLF AND ERLANGER's paper (1941) became available, in which they reported the results of similar experiments on single nerve fibres. Their results are in general consistent with ours, except that in theirs cold increased the spike height instead of decreasing it. This discrepancy seems to be fully accounted for by change in the resistance of the inactive tissues which surround the fibre under observation in their preparation.

It has been shown by YAMAGIWA (1935) that the size of the action potential from a single nerve fibre is markedly augmented by an increase in the resistance of the surrounding fluid and tissue. The action potential of the nerve is determined by the resistance of the inter-electrode portion of the nerve and the currents produced by individual

nerve fibres. Thus an increase of the nerve action potential by cold seems to attest to a decreased conductivity of the interelectrode portion of the nerve. Cold increases, as is well-known, the specific resistance of the electrolytic solution; but its effect upon the connective tissue sheath of the nerve is not known yet.

SCHOEPFLE AND ERLANGER investigated further the effect of cold upon the duration of the ascending and descending phases of the action potential. In their experiments, however, the inter-electrode distance is long and several nodes of RANVIER are involved between the two lead electrodes. An action potential record obtained in such circumstances should arise by virtue of the currents flowing between these nodes, but a direct comparison of these results with our data is not very easy at present.

Nervous conduction is a process intrinsic to the nerve fibre, and therefore the conduction rate does not vary significantly according to the method of measuring it. There is a remarkable agreement among the data, old and new, on the effect of temperature on the conduction rate. The temperature coefficient obtained by LUCAS (1908) is just the same as that obtained by use of the single fibre technique. The duration of the refractory period seems to depend to some extent upon the experimental arrangement adopted, but still almost all investigators give approximately the same absolute values and the same temperature coefficient. The threshold and the chronaxie are influenced greatly by the method of measurement, and consequently the agreement among the data of different investigators is not very good.

#### APPENDIX

##### 1. *The threshold for the exponentially rising voltage*

The time course of an exponentially rising voltage can be described by the formula

$$V(t) = V(1 - e^{-t/rc}),$$

where  $V$  stands for the final voltage and  $rc$  for the time constant of voltage rise. The time  $z$  at which the stimulating voltage crosses the rheobase  $b$  is given by the equation

$$b = V(1 - e^{-z/rc}). \quad (1)$$

The rate of voltage increase at this moment is given by

$$\left( \frac{dV(t)}{dt} \right)_{t=z} = \frac{V}{rc} e^{-z/rc}.$$

This rate must be greater than, or at least equal to, the minimal gradient  $m$  of the fiber in order that excitation can occur. Thus, for a threshold excitation,

$$V \frac{1}{rc} e^{-z/rc} = m. \quad (2)$$

Eliminating  $z$  from equations (1) and (2), we obtain the formula

$$V = mrc + b \quad (3)$$

which is fully verified by experiment.

*References p. 508/509.*

## 2. The excitability change for a rectangular current pulse

We shall denote the "latent addition curve" (the continuous line in Fig. 2 B or C) by the equation

$$Q = \varepsilon + q \cdot \psi(t), \quad (4)$$

where  $q$  represents the quantity of electricity of the conditioning shock and  $Q$  the threshold (quantity) for the test shock. This curve extends over the negative side of the time axis as far as  $t = -a$ , owing to the delayed maximum of the excitatory state mentioned in the text.

When a series of conditioning shocks, of which the quantities are given by  $q_1, q_2, q_3, \dots$ , are applied to the nerve fibre in succession at times  $t = 0, d_1, d_2, \dots$ , then the threshold for the test shock applied at time  $t$  is given, according to the law of superposition established by a previous experiment (TASAKI, 1942), by the equation

$$Q = \varepsilon + q_1 \cdot \psi(t) + q_2 \cdot \psi(t - d_1) + q_3 \cdot \psi(t - d_2) + \dots \quad (5)$$

A continuous constant voltage  $v$  may be regarded as a succession of brief voltage pulses having a quantity  $v dx$ . Then the contribution to the threshold from the voltage pulse bounded by  $t = x$  and  $t = x + dx$  should be given, as in the preceding case, by  $v \cdot dx \cdot \psi(t - x)$ . As all the elements of pulse between the time zero (at which the voltage starts) and  $t + a$  contribute to the variation of threshold at the time  $t$ , the value of  $Q$  at  $t$  is given by

$$Q = \varepsilon + v \int_0^{t+a} \psi(t-x) dx.$$

By the substitution  $y = t - x$ , we finally obtain the relation

$$Q = \varepsilon + v \int_{-a}^t \psi(y) dy. \quad (6)$$

The area under the latent addition curve is defined by

$$\varphi \equiv \int_{-\infty}^{\infty} \psi(y) dy = \int_{-a}^{\infty} \psi(y) dy.$$

For a sufficiently great value of  $t$ , equation (6) assumes the form

$$Q = \varepsilon + v\varphi. \quad (7)$$

This equation represents the linear relation between  $Q$  and  $v$  in the experimental results of Fig. 3, right. The ordinate  $S$  in Figs 2 and 3 corresponds to  $Q$  divided by the duration of the test shock.

## SUMMARY

1. An exponentially rising current excites an isolated single nerve fibre when, and only when, it rises above and crosses the rheobase at a rate of rise greater than the minimal gradient of the fibre. The rheobase is not affected by changes in temperature. The minimal gradient shows a tendency to be increased by cold, but there is a marked hysteresis in the effect of temperature changes on the minimal gradient.

*References p. 508/509.*

2. A brief subthreshold shock produces an excitatory state which first rises and then falls after the end of the shock. The time course of this process is retarded by cold. The minimum quantity of electricity needed to excite a single nerve fibre is increased by cold, its temperature coefficient being 1.7 for a change of  $10^{\circ}\text{C}$ .

3. At every temperature, the earliest return of excitability occurs immediately after the end of the spike. The process of recovery is affected by temperature changes to the same extent as the spike duration.

### RÉSUMÉ

1. Un courant croissant exponentiellement excite une fibre nerveuse isolée lorsque, et seulement lorsque ce courant dépasse la rhéobase à une vitesse de croissance supérieure à celle du gradient minimum dans la fibre. La rhéobase n'est pas modifiée par les changements de température. Le gradient minimum montre une tendance à augmenter par le froid, mais il y a une hysteresis notable dans l'influence des changements de température sur le gradient minimum.

2. Un bref choc sous-liminaire produit un état d'excitation qui d'abord croît, puis diminue après la fin du choc. La durée de ce phénomène est retardée par le froid. La quantité minimum d'électricité requise pour exciter une seule fibre nerveuse est augmentée par le froid, le coefficient de température étant de 1.7 pour un changement de  $10^{\circ}\text{C}$ .

3. A chaque température, le début du retour de l'excitabilité a lieu immédiatement après la fin du "spike". Le processus de récupération est affecté par les changements de température de la même façon que la durée du "spike".

### ZUSAMMENFASSUNG

1. Ein exponentiell wachsender Strom erregt einen isolierten einzelnen Nerv dann, und nur dann, wenn er die Rheobase übersteigt und mit einer Steigungsgeschwindigkeit kreuzt die grösser ist als das Mindestgefälle im Nervenstrang. Die Rheobase wird durch Temperaturänderungen nicht beeinflusst. Das Mindestgefälle zeigt die Tendenz, durch Kälte vergrößert zu werden, jedoch besteht eine beträchtliche Hysteresis in der Einwirkung von Temperaturänderungen auf das Mindestgefälle.

2. Ein kurzer unterschwelliger Schock bewirkt einen Erregungszustand welcher nach Ende des Schocks zuerst wächst und dann abnimmt. Der Zeitverlauf dieses Prozesses wird durch Kälte verlangsamt. Die zur Erregung eines vereinzelt Nervenstrangs benötigte Mindestmenge von Elektrizität wird durch Kälte vermehrt; der Temperaturkoeffizient beträgt 1.7 für eine Änderung von  $10^{\circ}\text{C}$ .

3. Bei jeder Temperatur findet die früheste Rückkehr der Erregbarkeit sofort nach dem Ende der "Spitze" statt. Der Erholungsprozess wird durch Temperaturänderungen in gleichem Ausmass beeinflusst als die "Spitzen"-Dauer.

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## THE STRUCTURE OF JUTE

## I. THE TWO-FOLD FUNCTION OF LIGNIN

by

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Not much systematic work has been done on the molecular structure of the bast fibres generally, although ramie has attained the status of a standard reference fibre in cellulose research. Such fibres as ramie and flax, however, are rather exceptional in that they are relatively pure cellulose; more usually the bast fibres contain high proportions of non-cellulosic inclusions, whose presence raises many important questions about the structure. In general it is safe to say that to a first approximation the structure of the crystalline fraction, as far as can be seen from the X-ray photographs, is much the same as in the purer cellulose fibres, and that therefore the non-cellulosic inclusions must exist, for the most part, in a non-crystalline condition. Some reservation with regard to the "hemicelluloses"—xylan and polyuronides—is, however, necessary in this connection, for their chain-molecules are sufficiently like those of cellulose to render plausible the suggestion<sup>1</sup> that they may in part be incorporated with the cellulose in a kind of mixed crystallization, without producing more than some slight distortion of the cellulose lattice. The other principal non-cellulosic constituent of the bast fibres, lignin, does not stand in any such close molecular relationship to cellulose, and its location is therefore almost certainly in the intercrystalline regions. It is the lignin in jute that we shall be chiefly concerned in this paper.

*Raw jute*

The X-ray photograph of raw jute is chiefly due to the crystalline cellulose in the fibre, and its main feature is that the reflections are more diffuse laterally than in ramie or cotton, so that, for example, the (101) and (10 $\bar{1}$ ) reflections are not resolved even in photographs of moderate photographic density. Longitudinally, the reflections in jute are of comparable sharpness with those in ramie, and the intensities of the meridional reflections (OkO) are very much greater in jute than in ramie. Both ramie and jute photographs show an intense equatorial streak extending inwards from the (101) and (10 $\bar{1}$ ) arcs and an even more intense equatorial small-angle scattering corresponding to spacings greater than 40 Å; these are rather more noticeable in jute. In the jute photograph there is also a darker background, and in particular a broad diffuse ring of mean spacing 4.2 Å which has been ascribed to lignin<sup>2, 3</sup>.

In jute photographs of moderate density it is possible to see quite clearly two diffraction maxima in the equatorial streak; the spacings are 9.7 Å (X) and 14.6 Å (Y),

*References p. 517.*

and Y appears to be stronger and sharper than X. The first layer-line in both ramie and jute photographs tends to show as a continuous streak from the meridian to (011), and this effect is more pronounced in jute. Asterism is generally better seen in ramie, but at the position where one radial streak in ramie crosses the first layer-line (at a spacing of approximately 8.5 Å) there appears in jute a clearly defined reflection (Z), even though the corresponding asterism streak may be too weak to be seen. Careful examination of the photographs of a number of pure and impure celluloses have shown that reflections similar to X and Y are of frequent occurrence, but that the intensities are generally very much smaller in the purer samples, and they are best seen in the photographs of such materials as jute, sawdust and bamboo (for information about the last named fibre we are indebted to PRESTON AND SINGH<sup>4</sup>). R. R. MUKHERJEE, working in this laboratory, has recently shown that the intensity of Y, at least, is greatest in wet fibres, and that the reflection is absent from the photograph of fibres dried over phosphorus pentoxide; he has also succeeded in treating ramie fibres in such a way as to enhance the intensity of Y to a degree which makes it comparable with that in jute. Our own observations, made on jute under ordinary conditions of atmospheric humidity, suggested that X and Y were due in some way to the hemicellulose, since they become more diffuse when the jute is treated with alkali (boiling  $\text{Na}_2\text{CO}_3$  or cold dilute caustic soda solutions), and though present in completely delignified jute (CROSS AND BEVAN cellulose) disappear when this is boiled in 7.5% caustic soda solution. The later results mentioned above, however, indicate that cellulose and water are both involved in the complex responsible for Y, and it is possible that the more frequent occurrence of the reflections in the "impure" fibres is due not so much to the presence in them of larger quantities of non-cellulosic materials as to the greater proportion of cellulose chains not taking part in the building of the crystallites and therefore available for forming a swelling compound with water. Since work on the subject of the new reflections is still proceeding, we shall defer any further consideration of this topic until this is completed.

### *Delignification*

Some clarification of the X-ray photograph usually accompanies the removal of lignin from highly lignified materials<sup>5</sup>. In jute we have observed a slight weakening of the background scattering and of the equatorial streak; but the most noticeable effect of delignification is that the diffuse ring at 4.2 Å appears to sharpen a little when the lignin content is reduced to some 2% whilst its intensity is reduced by further delignification. In some of our photographs the reflection seems to sharpen into a ring at 4.0 Å, but since this spacing nearly coincides with the (002) of cellulose it is impossible to be sure that the observed effect is not due to angular dispersion of the cellulose crystallites. In these experiments delignification was carried out by treating the fibres in  $\text{ClO}_2$  solutions (0.25 to 1.0%) for various times at 22.2° C, followed, after washing, by extraction in 2% sodium bisulphite solution at 90° C for 1 hour.

Powder photographs of lignin-free CROSS AND BEVAN cellulose<sup>6</sup> from jute were also cleaner in respect of the background than raw jute photographs, but by far the greatest improvement we have observed is the very pronounced change in clarity and cleanliness of the photograph brought about by boiling the CROSS AND BEVAN cellulose in 7.5% caustic soda solution. This treatment leaves the (101) and (10 $\bar{1}$ ) reflections clearly resolved and reduces the intensity of the background, in each case to an extent which

invites comparison with cotton cellulose, although even now the jute falls short of the perfection of the latter.

### *Mercerization*

The main features of the X-ray photograph of mercerized raw jute are that the native-hydrate transformation never appears to proceed to completion\*, and that the (101) hydrate spacing may have values very much greater than those observed in ramie or cotton. The reluctance of the last traces of the native cellulose to disappear from the photograph was established by examining jute after mercerization in caustic soda solutions of different concentrations at various temperatures. An attempt was made to estimate quantitatively the degree of modification by using the photometric method devised by SCHRAMEK<sup>7</sup>. This is based on measurements of the relative reflecting powers of the native and hydrate modifications in cellulose, and there is some doubt as to the validity of their application to jute; the results we give, therefore, must be considered with some reserve as to their accuracy, but they are adequate to establish the essential qualitative features of the phenomena. In Table I are shown the observed proportions of hydrate for jute mercerized without tension in caustic soda solutions of various concentrations at room temperature; in Table II the effect of temperature is shown for 20% and 30% caustic soda solutions. Measurements on jute fibres held taut during the

TABLE I

Concentration of caustic soda (%)	% hydrate
10	5
12	59
16	80
20	84
30	77

TABLE II

Temperature °C	% hydrate	
	20 % NaOH	30 % NaOH
0	74	84
25	83	77
40	85	88
80	84	91
100	81	86

treatment confirmed that in jute, as in the purer cellulose fibres, the effect of tension is to reduce the mercerizing action of the alkali, and that to an extent far greater in jute than in ramie, comparable figures for the proportions of hydrate being 5% and 75% respectively for jute and ramie treated with 20% caustic soda solution at 25° C.

It will be convenient to describe the behaviour with respect to the value of the (101) hydrate spacing in connection with the mercerization of delignified jute.

### *Mercerization of delignified jute*

Removal of the bulk of the lignin from jute has little effect on the course of the native-hydrate transformation, as judged from the observed proportions of the two modifications present. The results are set out in Table III, and it will be clear from these that it is only when the delignification is nearing completion that the proportion of hydrate rises significantly above the value observed for raw jute.

\* In this connection we may refer to a paper by SAHA (*Indian J. Physics*, 22 (1948) 141) in which he claims to have obtained complete mercerisation of raw jute under various conditions. We have not been able to confirm these findings in further experiments, the details of which will be published in due course. — H. J. W.

The effect of delignification in the early stages is better shown by the results of experiments designed to test the effect of water adsorption on the value of the (101) spacing. That there are lattice changes accompanying water adsorption by mercerized cellulose has been established by HERMANS AND WEIDINGER<sup>8</sup>, who report an increase from 7.3 Å to 7.7 Å in the (101) spacing of mercerized ramie when the fibres are conditioned at 65% R.H. after thorough drying. SIRKAR AND SAHA<sup>9</sup> have observed what appears to be an even greater effect in mercerized jute, their value at room humidity being 7.96 Å. We have investigated the phenomenon for raw jute and for jute delignified to various extents.

In mercerized raw jute, conditioned at 65% R.H. by *desorption* after washing free from alkali, that is, in fibres which have not been thoroughly dried after the alkali treatment, we obtain a spacing of 8.3 Å. This decreases as the fibres are dried further, reaching the value 7.4 Å for fibres dried over phosphorus pentoxide<sup>14</sup>. When the jute is now conditioned at 65% R.H. by *adsorption*, the spacing rises only to 7.7 Å, so that in this respect jute behaves like ordinary cellulose.

Similar measurements have been made for jute samples delignified to various degrees. At room humidity during the first drying the (101) spacing decreases with decreasing lignin content and reaches a minimum of 7.4 Å when the amount of lignin in the fibre has been reduced to about 2%, as may be seen from Table III. Beyond this stage the spacing rises again until it attains approximately the normal cellulose value when the lignin has been entirely removed.

The effect of complete drying has been investigated for jute containing 6% of lignin and also for CROSS AND BEVAN cellulose from jute. In both, the hydrate spacing of specimens dried over phosphorus pentoxide has the same value, 7.4 Å, as in the

TABLE III

Lignin (%)	% hydrate	(101) spacing (Å)
0.0	100	7.6 <sub>7</sub>
0.3	91	7.5 <sub>5</sub>
2.0	85	7.4 <sub>0</sub>
6.0	81	7.4 <sub>5</sub>
9.0	83	7.6 <sub>0</sub>
11.0	86	7.8 <sub>5</sub>
Raw jute	84	8.3 <sub>0</sub>
Ramie	100	7.8 <sub>5</sub>

corresponding experiment with raw jute. It appears, therefore, that in the region where values near 7.4 Å are obtained spontaneously at room humidity during the first drying the effect of water in causing intra-crystallite lattice changes is practically eliminated, whilst the behaviour of CROSS AND BEVAN cellulose agrees closely with that of pure cellulose fibres.

#### DISCUSSION

The results set out in Table III make it clear that the removal of lignin from jute can be divided into two stages; during the first of these the larger part of the lignin comes away without leaving the jute any more susceptible to the mercerizing action of caustic soda solutions, whilst the second stage involves the removal of that lignin which is

chiefly responsible for the non-completion of the native-hydrate transformation. The failure of jute containing more than a trace of lignin to mercerize completely must be ascribed to a depression of the swelling in caustic soda solutions, since it is generally accepted that successful mercerization is a consequence of a sufficiently high degree of swelling. One effect of the lignin in jute, therefore, is to reduce the swelling in caustic soda solutions, and it is the lignin which is most difficult to remove which is effective in this respect.

This, however, is not the only way in which the presence of lignin modifies the course of the mercerization process, for Table III shows also that there is an even more complicated relation between the lignin content and the value of the (101) hydrate spacing on first drying the fibres at room humidity after the alkali treatment. In particular, the spacing is exceptionally high in raw jute, and becomes smallest in the region where the proportion of the hydrate modification starts to increase. The course of the native-hydrate transformation is known to proceed through a series of recrystallizations from soda-cellulose through water-cellulose to the true cellulose II. These intracrystalline readjustments are characterized by changes in the (101) spacing, which in water-cellulose has the value  $8.98 \text{ \AA}^{10}$  and in cellulose II  $7.3 \text{ \AA}$  increasing to  $7.7 \text{ \AA}$  with adsorption of water<sup>8</sup>. It is not known whether the transition from water-cellulose to cellulose II is continuous in respect of the (101) spacing changes\*, but we have observed, even in ramie, the hysteresis between the spacing assumed at room humidity during the first drying and that shown on conditioning by adsorption. The effect, however, is overwhelmingly greater in jute, so much so that the hypothesis of a continuous transition from water-cellulose to cellulose II becomes plausible; at any rate, we can describe the phenomenon in jute as a lag in the process of intracrystalline readjustment as the water is removed from the water-cellulose. Since the observed (101) spacing in mercerized jute depends on the lignin content we can ascribe this reluctance to assume the cellulose II configuration to the influence of the lignin, and in this respect it is the more easily removed lignin which is responsible. It is relevant in this connection to note that concurrently with the spacing changes noted in Table III we have observed corresponding changes in the sharpness of the (101) spacing, which improves as the value of the spacing decreases, so that there is some justification for describing the transition as a crystallization into the cellulose II form.

We are thus led, by consideration of the mercerization phenomena, to the idea that lignin can perform a double function in jute. In the first place it can hinder the swelling in the caustic soda solution, thus preventing the quantitative completion of the native-hydrate transformation, and in the second place it can, by its very presence, interfere with the packing together of the cellulose chains which is necessary for the development of perfect crystallinity. Although we have considered this latter effect as it appears in connection with the mercerization phenomena, it is possible that it might also be a contributory factor to the imperfect crystallinity of raw jute. For we know that lignin is distributed throughout the body of the jute cell (although the concentration is greater in the regions of the cell boundaries, at the middle lamella and near the lumen), so that part of the lignin can be thought of as existing as a kind of solid solution in the non-crystalline cellulose and hemicellulose. Since the latter must be assumed to form a continuum with the crystalline regions, any influence tending to

\* Later results, for which we are indebted to R. R. MUKHERJEE, show that the spacing changes in jute are continuous.

keep the chains apart and thereby restricting their freedom to fit together into a state of higher organization, even if limited in the first instance to the non-crystalline regions, must extend, by virtue of the structural continuity, into the crystallites themselves, which will therefore show less perfect crystallinity than would exist in the absence of the disturbing influence. Although we mention this possibility here, it must not be thought that we thereby deny the validity of the hypothesis put forward by ASTBURY, PRESTON AND NORMAN<sup>1</sup> that the imperfect crystallinity in jute and other fibres with high lignin and hemicellulose contents is largely due to the incorporation within the cellulose crystallites of some portion of the hemicelluloses in a state of mixed crystallization; we have, in fact, evidence, which we shall bring forward in a later paper, which offers strong support for this idea. We cannot, however, subscribe to NORMAN's later interpretation<sup>11</sup> of the original results, which implies that this mixed crystallization accounts for the whole of the hemicelluloses of the hexosan or pentosan types, whilst the polyuronide hemicelluloses and the lignin are incrustations existing in aggregations in the larger pores of the cellulose — cellulosan structure.

A further consequence of the presence of lignin in the non-crystalline regions of the fibre, where by steric hindrance it modifies the packing together of the cellulose chains, would be that in this respect the lignin would exert an influence in direct opposition to the anti-swelling tendency which we have had to postulate for the most tightly-held lignin. For the greater separation of the chains in the non-crystalline regions would lead to easier penetration of water or other swelling agents on account of the more open structure, and it might be possible on these lines to account for the fact that jute is much more hygroscopic than the purer cellulose fibres, although it has not been reported to show greater swelling in water. This idea would also fit in with the observations which we have made of the lattice changes consequent upon water adsorption by mercerized jute. We find that the value of the (101) spacing in perfectly dry fibres does not depend on the lignin content, and we can say that the intracrystalline readjustments which take place when both raw jute and completely delignified jute absorb water are practically eliminated, at least in the first part of the adsorption process, in jute containing a few per cent of lignin. At this stage in the delignification, therefore, only the anti-swelling influence of the residual lignin can be effective. In the last stages of delignification the lattice changes are again apparent in consequence of the removal of the final traces of lignin, which are chiefly responsible for the depression of the swelling.

It might, perhaps, be argued that the potentiality for increased swelling which results from the removal of the last few percent of lignin is due to some process of degradation of the cellulose itself as a consequence of the comparatively drastic treatment which the fibres have undergone. This argument must be rejected, however, since it does not account for the swelling behaviour of normal and moderately delignified jute in caustic soda solutions. To explain this it seems necessary to suppose that delignification must be regarded as removing some restrictive influence which prevents the full swelling of the cellulose-hemicellulose complex. It is tempting to speculate on other possibilities which are in conformity with this idea. We can imagine, for instance, that the highly lignified regions at the cell boundaries form a sort of envelope enclosing the bulk of the cell wall, and that this lowers the swelling by mechanical restriction<sup>13</sup>. If the distribution of lignin retains its essential non-uniformity during the course of the delignification it would be reasonable to suppose that the lower swelling would persist until a stage is reached where the removal of lignin from these highly lignified regions

leaves them incapable of exerting their restrictive action. Some support for this hypothesis comes from the observation that the diffuse DEBEYE-SCHERRER ring at  $4.2 \text{ \AA}$  on the X-ray photograph of jute, which has been attributed to lignin, persists until the lignin content is less than 2%; for we might expect the lignin which is sufficiently aggregated to give rise to an X-ray reflection, however diffuse, to be located in the most highly lignified regions of the fibre. Staining tests also indicate that the lignin near the lumen, at least, is rather difficult to remove. On the other hand LANGE<sup>12</sup> reports that in wood there is, during the early stages of delignification, a considerable removal of lignin from the middle lamella, although his published results do not exclude the possibility that the boundary concentrations should persist, in part at least, until the last stages.

The other possibility which we may consider is that the last lignin to be removed is combined in some way with the non-crystalline cellulose or hemicellulose chains which are located near the crystallites, forming, perhaps, bridges between them and thus reducing intercrystalline, and therefore intracrystalline, swelling. That this lignin should be the most difficult to remove would follow from the fact that it is so combined; and that there is some chemical combination between lignin and the hemicelluloses, at least, has been often conjectured.

It is clearly impossible to decide, on the evidence available, if either of these possibilities is correct. Further work, which it is hoped will clarify the position, is in progress, and will be reported on in a later paper in this series.

#### SUMMARY

1. There appear at relatively high intensity in the X-ray photograph of jute certain reflections not due to the accepted cellulose lattice, which are normally only seen with difficulty, if at all, in the purer cellulose fibres. In particular, one of these, an equatorial reflection of spacing  $14.7 \text{ \AA}$ , occurs not only in jute but in a wide range of other plant fibres; its intensity is strongly dependent on the water content of the fibres, which suggests that it is due to some sort of swelling compound between water and either cellulose or hemicellulose chains which is sufficiently regular, laterally at least, to give rise to X-ray reflections.

2. Both the diffuseness of the cellulose reflections, and to some extent the background scattering, in the X-ray photograph of jute are associated with the presence of lignin and the hemicelluloses. Removal of both types of inclusion can increase the degree of perfection of the crystallinity of the cellulose to a level approaching, but not reaching, that of cotton. Removal of either type alone has relatively little effect.

3. The presence of lignin can a) act as a swelling depressant, and so impose a limitation of the intra-crystalline swelling necessary for the transformation into the hydrate modification; and b) by its presence between the cellulose and hemicellulose chains hinder any tendency on their part to assume a state of higher organization. Thus the extent of mercerization of jute in caustic soda solutions, the intra-crystalline swelling of the hydrate modification, and the sharpness of the reflections in the hydrate photograph all depend on the lignin content.

#### RÉSUMÉ

1. Des photographies faites avec des rayons X montrent dans le jute certaines réflexions qui ne sont pas dues aux réseaux connus de la cellulose, et qui ne sont visibles qu'avec beaucoup de difficulté, ou même ne sont pas visibles du tout dans les fibres de cellulose pure. En particulier, l'une de ces réflexions, équatoriale, correspondant à un espace de  $14.7 \text{ \AA}$ , se rencontre non seulement dans le jute, mais chez une série d'autres fibres végétales; son intensité dépend dans une large mesure de la teneur en eau des fibres, ce qui conduit à penser qu'elle est due à un système résultant de l'imbibition par l'eau soit de cellulose, soit d'hémicellulose, dont l'arrangement est suffisamment régulier pour donner naissance à des réflexions des rayons X.

2. Le degré de diffusion de réflexion de la cellulose, et le flou du fond de la photographie du jute par les rayons X, sont dus à la présence de lignine et des hémicelluloses. L'élimination de ces deux types de substances permet d'accroître la purification de la cristallinité de la cellulose, jusqu'à l'approche de celle du coton. L'élimination d'un des types seulement n'a guère de résultats.

*References p. 517.*

3. La présence de lignine peut: a) agir comme un inhibiteur du gonflement et ainsi limiter le gonflement intracristallin nécessaire pour la transformation en l'hydrate; b) par sa présence entre les chaînes de cellulose et d'hémicellulose, les empêcher de prendre un état d'organisation plus élevé. Ainsi, le degré de mercerisation du jute dans une solution de soude caustique, le gonflement intracristallin de l'hydrate et la netteté des réflexions obtenues par photographie de l'hydrate, tout ceci dépend de la teneur en lignine.

#### ZUSAMMENFASSUNG

1. Photographische Aufnahmen von Jute mit Röntgenstrahlen zeigen gewisse Reflexionen, welche nicht von dem bekannten Zellulosegitter stammen und die in reinen Zellulosefasern nur schwer oder garnicht sichtbar sind. Insbesondere eine äquatoriale Reflexion, welche einem Abstand von 14.7 Å entspricht, tritt nicht nur bei Jute auf, sondern auch bei anderen Pflanzenfasern; ihre Intensität hängt stark vom Wassergehalt der Fasern ab, was darauf schliessen lässt, dass sie von einer durch Quellung der Zellulose- oder Hemizelluloseketten entstandenen Verbindung herrührt, deren Anordnung, zumindest lateral, genügend regelmässig ist, um Reflexionen der Röntgenstrahlen zu bewirken.

2. Die unscharfen Reflexionen und der verschwommene Hintergrund der Röntgenstrahlenaufnahmen von Jute rühren vom Lignin und den Hemizellulosen her. Entfernt man diese beiden Arten von Einschlüssen, so kann man die Zellulose nahezu so rein und kristallin erhalten, wie sie in der Baumwolle vorliegt. Entfernt man nur die eine oder andere Art, so ist das Ergebnis gering.

3. Das Lignin kann entweder: a) die Quellung hemmen und so die zur Bildung des Hydrates nötige intra-kristalline Quellung eindämmen, oder b) durch seine Lage zwischen den Zellulose- und Hemizelluloseketten, deren etwaige Neigung in einen Zustand höherer Organisation überzugehen, hindern.

Die Mercerisierung der Jute in Natronlauge, die intra-kristalline Quellung des Hydrates sowie die Schärfe der Reflexionen in photographischen Aufnahmen des Hydrates hängen also alle vom Ligningehalt ab.

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## NOTE ON SPIRAL GROWTH AND SPIRAL CELL WALL STRUCTURE IN SPORANGIOPHORES OF *PHYCOMYCES*

by

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Recently a very attractive hypothesis has been postulated in this journal by R. D. PRESTON (1948) on the spiral growth of *Phycomyces* sporangiophores, which would be governed by the spiral structure of the primary cell wall in the growth zone of these cells. The rotation of the sporangium, or of the tip in young sporangiumless cells, is compared with the rotation of the top of a flat spiral wire which is being extended by some pulling force.

Although this line of interpretation might not be too far removed from the truth, the present author is of the opinion that in applying this hypothesis to this object certain facts have been overlooked.

The usually predominating *left-handed*\* spiral of growth is explained by PRESTON by *left-handed* structural spirals in the (primary) cell wall of the growth zone, thereby however neglecting two facts, *viz.*:

a) the optical work of OORT AND ROELOFSEN (1932) indicates a flat *right-handed* structural spiral;

b) a *left-handed* spiral does not fit his spring-elongation theory in explaining a clockwise rotation of the sporangium, as elongation of such a spiral would cause a counter-clockwise rotation.

As to a) the following may be remarked. PRESTON (*l.c.*, p. 161) states:

"In actual fact, the optical work which has been done on the wall suffices only to indicate that the chains are inclined to the transverse at angles considerably less than 45°. It is not clear in the literature even whether the spiral (1)\*\* is left- or right-handed.

It is stated by OORT AND ROELOFSEN (1932) that on the whole the spiral (2)\*\* is *left-handed*, and on the drawing they give (which shows, however, a right-hand spiral) (3)\*\* the angle drawn is about 8° for what that is worth".

Here the results of OORT AND ROELOFSEN (*l.c.*) are evidently misinterpreted, because in Fig. 1 of their publication, reproduced here again as Fig. 1, the right handed spiral (spiral no. 3 of PRESTON), marked *ny*, in cell-wall layer 1, represents only the spiral of the *highest refractive index* and neither the spiral of the chitin chains (spiral no. 1 of PRESTON) nor the spiral of growth (spiral no. 2 of PRESTON).

\* Left-handed in the English sense, *i.e.*, in descending a spiral the axis will be on the left hand. With a left-handed spiral of growth the sporangium rotates clockwise when looking down on it.

\*\* Numbered by present author.

In summarizing, OORT AND ROELOFSEN (*l.c.*) stated:

"In der primären Wand (Schicht 1 ist in der Wachstumszone allein vorhanden), stimmt die Richtung der grössten Dehnbarkeit, der Spaltbarkeit, einer feinen Streifung und von  $n\beta$  mit der Wachstumsrichtung überein. Diese verläuft meistens in einer steilen Rechts-\*, bisweilen auch Linksspirale.  $n\gamma$  steht senkrecht auf dieser Richtung und verläuft ungefähr tangential".

So here it was pointed out, that the spiral of growth (Wachstumsrichtung) was left-handed whereas  $n\gamma$  was right-handed. A mistake, as supposed by PRESTON, was therefore excluded. OORT AND ROELOFSEN (*l.c.*) did not venture to take a decision concerning the direction of chitin strands, but mentioned (p. 905) the following theoretical possibilities:

1. the strands run parallel to  $n\gamma$  and consist of rodlets, also lying roughly parallel to  $n\gamma$ ;

2. the strands run perpendicular to  $n\gamma$ , but consist of piled-up platelets and therefore their textural birefringence is negative.

Now possibility 2 is very improbable and actually impossible with a view on the orientation of the indices of birefringence in transversal and radial sections of the primary cell wall. The only objection against assumption of possibility 1 is, that the preferred line of burst ("Spaltbarkeit" in citation above) was seen to be perpendicular to  $n\gamma$ , whereas one would expect it parallel to  $n\gamma$  and to the strands of chitin rodlets.

However before the sporangiophore is brought to burst by pressing on the basal portions, the top shows torsions accompanying the elastic elongation and the direction of the burst-line can very easily be wrongly interpreted. Keeping in mind that further investigations on this point are required, one may assume for the present possibility 1.

Concerning consideration b, mentioned above, it may be pointed out that a clockwise rotation of the top is shown, when a right-handed spiral of, for instance, steel wire is elongated, as can be seen from the difference in position of the tops in Figs 2 and 3, which actually represent drawings of models. PRESTON (*l.c.*, p. 162) states: "It is further to be noted that since  $n/q$  is less than unity then the spiral growth is left-handed (if the structural spiral is left-handed and since the spiral coils up on itself). This is the commonly observed direction of rotation".

With a steel wire  $n/q$  certainly will be less than unity, but it is observed that the spiral does not coil up on itself. Such would only happen if the rotation is *not* due to elongation of spirals, but only to growth in length of the strands constituting the spirals. Such growth is also assumed by PRESTON (*l.c.*, p. 159) when he states "We have here, then, a series of spirals which are kept in continuous elongation by the insertion of new elements".

Considering the optical facts as elucidated by OORT AND ROELOFSEN (*l.c.*) (essentially confirmed by CASTLE, 1938) and making use of the spiral-elongation-theory of

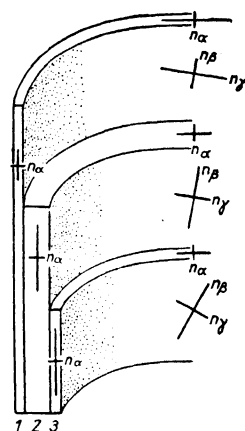


Fig. 1. Schematic representation of the cell-wall layer with refractive indexes inserted

\* Right and left here contrary to the English sense.

PRESTON, corrected as to the direction of the rotation, furthermore assuming that the chitin strands in the primary and the secondary wall run approximately parallel to  $\eta\gamma$ , as drawn in Fig. 1, the spiral growth can be easily explained as follows.

A. The *usual clockwise* rotation of the sporangium, in accordance with a left-handed spiral of growth, may be due to one or more of the following causes.

1. The flat *right-handed* chitin-strand spirals in the cell wall of the upper parts of the growth zone are elongated by turgor-tension, become steeper and cause a clockwise rotation, as explained by PRESTON's theory, see Figs. 2 and 3. In the meantime they become located in lower parts of the growth zone.

2. These spirals not only elongate, but also acquire a larger radius because the sporangiophore in the growth zone is not tubular, but somewhat cone-shaped. As demonstrated by Fig. 4, such enlargement of right-handed spirals also causes clockwise rotation of the top.

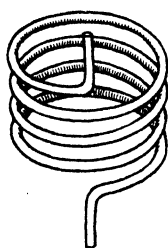


Fig. 2. Model of spiral steel wire

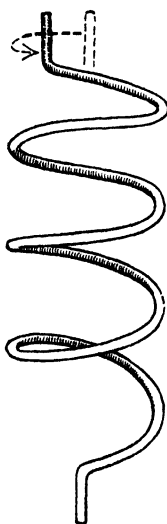


Fig. 3. Spiral of Fig. 2 elongated

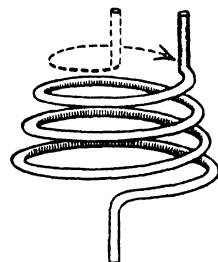


Fig. 4. Spiral of Fig. 2 enlarged coneshaped

3. When the spirals are elongated (or widened in radius) as supposed under 1 (or 2) the widening spaces between the "primary" chitin strands considered, very probably will be filled up, at least partly, with new cell-wall material. When the chitin-material is supposed to consist of strands, then what happens is an increase of the number of strands. As long as this intussusception of new "secondary" strands does not exceed the task of filling the required space, no additional rotation is originated. Only, if this would not take place, the rotation would be diminished by increasing elastic resistance, but in case *more* "secondary" strands are intussuscepted, a clockwise rotation will result of it. This would make the sporangiophore both wider and longer, dependent on the steepness of the structural spirals. It would therefore diminish the rotational forces due to 1 and 2.

B. *Counter clockwise* rotation of the sporangium may be due to one or more of the following reasons:

References p. 522.

1. PRESTON's theory concerning the reversal of the growth spiral when  $2n$  becomes greater than  $q$ .

2. Domination of the clockwise rotation by the counter-clock-wise rotational effect, caused by the growth in length of the strands of chitin chains constituting the spirals. Depending on how one supposes that the interpolation of chitin units in the chains takes place, one may speak of growth of the chains or of slipplanes between the chains, or between the strands (HEYEN, 1939). This process, whatever it may be, may be located for instance at the apical ends of the chains in the topmost part of the growth zone, or distributed over the entire growth zone. Due to this process "the spirals will coil up on themselves" and as they are right-handed a right-handed rotation of the sporangium will occur. Actually this counter-clockwise rotational influence will be constantly present during periods of clockwise rotation, but in insufficient degree to compensate the clockwise rotation.

The present author is inclined to believe that this process in fact causes the small counter-clockwise rotation occurring at the onset of growth after the sporangium has been formed (stage IVa of CASTLE, 1942). In stage III (CASTLE) the former growth zone has probably been stiffened by the deposition of (primary or secondary) cell wall material. In that case a new growth zone must be generated. In the initial phase of this, when no widening of the cell radius comes into question, intussusception is excluded, as well as all growth due to plastic elongation. Then growth in length of the chitin strands themselves is the only means of starting sporangiophore growth. The high quotient  $\frac{\text{rotation}}{\text{elongation}}$  in this stage, as found by CASTLE (*l.c.*), is in accordance with this assumption.

3. During a period of lowering of the turgor-tension the effect of the factors mentioned under A 1 and 2 will be reversed.

4. When temporary the secondary cell wall, which, as Fig. 1 shows, is probably constituted of steep left-handed structural spirals, is deposited in the growth zone, or at least in a zone which later on resumes growth, the factors mentioned under A 1 and 2 may be reversed if the secondary wall dominates the primary wall. (This may also explain the counterclock wise rotation in stage IVa).

#### SUMMARY

It is pointed out that the theory of PRESTON (*l.c.*) explaining spiral growth of *Phycomyces* sporangiophores by elongation of chitin strand spirals in the primary cell wall is fully in accordance with the known facts about its physical structure as described by OORT AND ROELOFSEN (*l.c.*). More possibilities causing spiral growth and temporary reversal of the spiral growth direction from left to right are however mentioned.

#### RÉSUMÉ

L'auteur démontre que la théorie de PRESTON (*l.c.*) expliquant la croissance en spirale des sporangiophores du *Phycomyces*, basée sur l'allongement des spirales de fascicules de chitine dans la paroi primaire, est en accord complet avec les faits décrits sur la structure physique par OORT ET ROELOFSEN (*l.c.*).

En outre, il propose quelques circonstances qui pourraient causer aussi cette croissance en spirale et son changement de direction temporaire (de gauche à droite).

References p. 522.

## ZUSAMMENFASSUNG

Es wird gezeigt, dass die Theorie von PRESTON (*l.c.*) die das Spiralwachstum des Sporangiphoren von *Phycomyces* durch die Verlängerung der spiralförmigen Bündel von Chitin in der primären Wand erklärt, vollkommen mit den von OORT UND ROELOFSEN (*l.c.*) beschriebenen Tatsachen über ihre physikalische Struktur übereinstimmt.

Es werden ausserdem noch andere Umstände erwähnt, die ebenfalls dieses Spiralwachstum und seine zeitweilige Richtungsänderung von links nach rechts bewirken könnten.

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## ULTRACENTRIFUGAL STUDIES OF F-ACTOMYOSIN

by

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NEEDHAM *et al.*<sup>1</sup> found that the viscosity of myosin solutions in 0.5 M KCl decreases if small amounts of adenosinetriphosphate (ATP) are added to the solutions. BANGA AND SZENT-GYÖRGYI<sup>2</sup> found that if the muscle is extracted for a longer time (24 hours) a highly viscous myosin preparation is obtained. If ATP is added to such a preparation the viscosity decreases considerably. STRAUB<sup>3</sup> showed that the high viscosity was due to the fact that another substance, "actin", went into solution with the myosin to form a highly viscous complex, actomyosin.

SZENT-GYÖRGYI *et al.*<sup>4</sup> have shown further that if dilute solutions of myosin and polymerized actin (F-actin) are mixed the mixture immediately shows a large increase in viscosity, indicating that the myosin and the F-actin have reacted in some way. They called the complex thus formed F-actomyosin. It is dissociated under certain conditions by the action of ATP, with an accompanying decrease of the viscosity.

Actomyosin has some of the properties of crystallized myosin and actin but also many new properties. We do not wish to go into all these differences, but will only mention the following: Actomyosin can be contracted, precipitated and dehydrated by the action of ATP in certain definite salt concentrations. These properties are the basis for considering actomyosin as the contracting substance in muscle. SZENT-GYÖRGYI has assumed, from the beginning, that no definite stoichiometrical relation exists between actin and myosin in F-actomyosin, although there is an optimal ratio of one part actin to 2.5 parts myosin.

The object of our experiments has been the investigation of the behaviour of actomyosin (also called actomyosin only) in the ultracentrifuge, and to try to find if actin and myosin react in a stoichiometrical ratio.

## ULTRACENTRIFUGAL ANALYSIS OF F-ACTOMYOSIN

F-actomyosin was prepared by mixing myosin and F-actin a few minutes before the experiment. F-actin was prepared by dialysing the unpolymerized actin (G-actin) over night at 4° C against the buffer solution that would be used, with the addition of 0.001 M MgCl<sub>2</sub>. Determinations of the viscosity were carried out at the same time as the ultracentrifugations. During the measurements the temperature was 20° C.

The first experiments were carried out with the oil turbine ultracentrifuge<sup>5</sup> at 30000 rpm (centrifugal field = 65 · 10<sup>3</sup> g). Most often two components were observed, the sedimentation constants of which were not considerably far apart. The sedimentation

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constants were very dependent upon the concentration. The extrapolated sedimentation constants (conc. = 0) were of the order 200 S. The concentration calculated from the sedimentation diagram was much too low as compared to that which could be expected.

The following experiments were carried out in the equilibrium centrifuge (a low speed ultracentrifuge with direct motor drive<sup>5</sup>) at 18000 rpm (centrifugal field =  $19 \cdot 10^3$  g) and 10000 rpm (centrifugal field =  $6 \cdot 10^2$  g).

It was hereby shown that the greater part of the actomyosin sedimented as a gel. Because this actomyosin gel sedimented so quickly it had not been detected in the runs at 30000 rpm, where it sedimented while the centrifuge was accelerating.

The gel formation seemed to be much greater with the synthetically prepared actomyosin than with that prepared directly from muscle by prolonged extraction.

Some experiments have also been carried out with actomyosin in the presence of ATP. In these experiments, 0.01 M  $MgCl_2$  and 1 g potassium salt of ATP were added to the actomyosin solutions. The solutions were buffered with veronal buffer to  $p_H$  6.8. The solutions contained 3 mg of myosin in 100 ml. The actomyosin usually splits ATP but under the experimental conditions used by us no such splitting of ATP takes place (temperature 20° C). Time of the experiment 2 hours. 0.01 M  $MgCl_2$  added to prevent the splitting).

The sedimentation diagrams show in the presence of ATP the components with the very high sedimentation constants disappear while the characteristic diagrams for myosin<sup>7</sup> and F-actin appear. (About the behaviour of F-actin in the ultracentrifuge will later be reported). The dissociation of F-actomyosin into these components clearly takes place under the conditions given.

#### INVESTIGATION OF THE RATIO F-ACTIN TO MYOSIN IN F-ACTOMYOSIN

The fact that we found in earlier experiments<sup>6</sup> that the solutions prepared by extraction of the muscle for 24 hours contained myosin and actomyosin indicates that a mixture of myosin and actin does not, as previously assumed, react to form an actomyosin of completely indefinite proportions. In order to investigate the question more closely ultracentrifuge and viscosity investigations were made with synthetic actomyosin prepared from the crystallized myosin and F-actin.

At first we tried to separate the substances by differential centrifugation. Myosin has a much lower sedimentation constant than actomyosin and F-actin, so no difficulties are encountered in its separation from the other substances. In these experiments the BEAMS preparative centrifuge was used. After the centrifugation the sediment was separated from the supernatant. The sediment was redissolved in the buffer and viscosimetric measurements according to STRAUB<sup>3</sup> were made of both the solutions.

From experiments with different amounts of actin added to a certain amount of myosin we found that 1) if myosin and actin are mixed in an optimal proportion (1 part actin to 3 parts myosin) the whole protein content sediments to the bottom as actomyosin and no protein is found in the supernatant (no cloudiness occurs with trichloroacetic acid); 2) if less actin is present than corresponds with the optimal ratio free myosin remains in the solution while the actomyosin sediments to the bottom; 3) if more actin is present than corresponds with the optimal ratio, free F-actin is found in the supernatant. The last experiment was carried out with a certain amount of difficulty, because the difference between the sedimentation constants of F-actin and F-actomyosin is not

very large. We would add that, in the case that myosin and actomyosin are both present, there are always traces of actomyosin in the supernatant. It is therefore not possible to separate myosin completely from actomyosin by this method.

We have also tried to determine the amount of myosin which is found in solutions containing actomyosin by adding varying amounts of F-actin to a specified amount of myosin, and by determining the myosin concentration from the sedimentation diagrams. The result of these investigations are seen from Table I.

TABLE I

DETERMINATION OF THE MYOSIN CONTENT FROM ULTRACENTRIFUGAL EXPERIMENTS WHEN DIFFERENT AMOUNTS OF ACTIN ARE ADDED TO A FIXED AMOUNT OF MYOSIN (3 mg/ml)

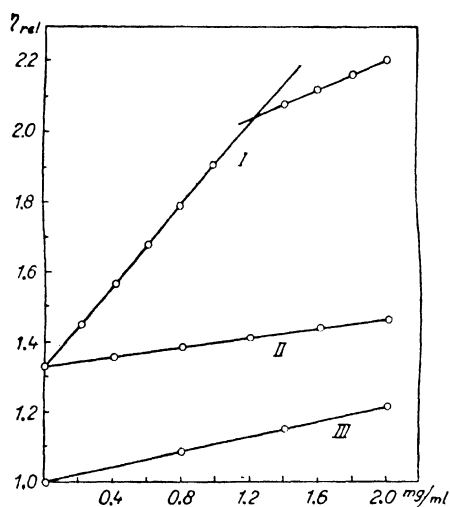
Added amount of actin in mg/ml	$s_{20}$	Amount of myosin calculated from the sedimentation diagram in mg/ml
0	5.7	3.0
0.2	6.0	2.4
0.4	6.1	1.8
0.6	6.6	1.2
0.8	+	Myosin component observable, but not measurable with certainty
1.0	—	Actin component visible in these two cases
1.2	—	

The values of the sedimentation constants agree with the values which we obtained in the study of the relation between concentration and sedimentation constant of the crystallized myosin<sup>7</sup>. The component can therefore be identified with myosin. The concentration decreases in well-defined equal steps as the actin content increases. It is clear that the actin binds a definite amount of myosin, and, when there is a deficiency of actin, part of the myosin is left in solution. At a certain actin concentration (optimal) no myosin is found in the solution and all the protein sediments as actomyosin. If more actin is added a new component appears which we could recognize as F-actin (other unpublished investigations).

The optimal proportion was in this case: 1 part actin to 3 parts myosin.

The problem was also studied viscosimetrically in order to see if it was possible, in this way, to determine the stoichiometrical proportions between actin and myosin when actomyosin is formed.

The same mixtures were used as in the ultracentrifuge experiments, except that the solutions were diluted to twice their volume. The relative viscosity measurements were carried out according to the method given by STRAUB<sup>3</sup>. The measurements were made at 20° C since the ultracentrifuge determinations were carried out



Viscosity curves. Ordinate: relative viscosity. Abcissae: F-actin added in mg/ml. I. Viscosity curve of a myosin solution with different amounts of actin added; II. Viscosity of the solution when ATP is added; III. Concentration dependence of the viscosity of F-actin



at that temperature. When the relative viscosity of a solution had been determined, 1 mg of ATP was added and the measurement repeated. The measurements were completed by measuring the relative viscosity of a series of actin solutions of different concentrations. The results of the measurements are given in Fig. 1. Since the solutions show thixotropy the measurements were repeated until constant values were obtained.

The relative viscosity of the mixtures is increased by an increased amount of actin. The curve is linear and has a break at a certain amount of actin indicating that a change has taken place in the proportions at this point. After the break the relative viscosity increases very nearly at the same rate as in the case of actin. If the discontinuity constitutes the endpoint of the formation of actomyosin, it corresponds to a compound containing 1 part actin to 2.5 parts myosin.

It appears from these measurements that myosin and actin combine very nearly stoichiometrically. Both methods used are subject to certain errors whereby an absolute agreement is not to be expected.

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#### SUMMARY

F-actomyosin has been investigated in the ultracentrifuge. It is a polydisperse substance. Two different main components are visible. One fraction has a very pronounced gel-like character and sediments very quickly. The other fraction shows a sedimentation picture characteristic of large long-chain molecules.

Experiments carried out indicate that actomyosin is a stoichiometric compound of actin and myosin. The values obtained from ultracentrifuge and viscosity data indicate that actomyosin contains 1 part actin to 2.5-3 parts myosin.

#### RÉSUMÉ

La F-Actomyosine a été étudiée à l'ultra-centrifugeuse; c'est une substance polydisperse. Deux principaux composés différents peuvent être observés. Une fraction possède un caractère très prononcé de gel et se sédimente très rapidement; l'autre fraction montre une sédimentation caractéristique des molécules à longues chaînes. Les expériences faites montrent que l'actomyosine est un composé stoechiométrique de l'actine et de la myosine. Les valeurs obtenues à partir des résultats de l'ultra-centrifugation et des mesures de viscosité indiquent que l'actomyosine contient une part d'actine pour 2.5 à 3 parts de myosine.

#### ZUSAMMENFASSUNG

F-Aktomyosin wurde in der Ultrazentrifuge untersucht; es ist eine polydisperse Substanz. Man kann zwei verschiedene Hauptfraktionen unterscheiden, von denen die eine ausgesprochenen Gelcharakter hat und sehr rasch sedimentiert, während die andere das für lange Kettenmoleküle charakteristische Sedimentationsbild zeigt.

Die Versuchsergebnisse weisen darauf hin, dass Aktomyosin eine stöchiometrische Verbindung von Aktin und Myosin ist. Die Ergebnisse der Ultrazentrifugation und der Viskositätsmessungen lassen darauf schließen, dass Aktomyosin für je 1 Teil Aktin 2.5-3 Teile Myosin enthält.

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## THE STRUCTURE OF BACTERIAL CELLULOSE

by

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## INTRODUCTION

Electron microscopic studies<sup>1</sup> have shown that the cellulose from several different plants occurs as fibres of a diameter of ca 250 Å. How these fibres are built up and why they have so constant a diameter is still unknown and the investigation of such questions is made especially difficult by the fact that the cellulose is produced within the cells of these plants. They could be studied in thin section but the necessary techniques are not yet sufficiently developed to make this possible. It therefore has seemed more profitable to evade these difficulties by choosing for study an organism which builds cellulose extra-cellularly.

As long ago as 1886 A. J. BROWN<sup>2</sup> described a bacterium which produced a solid membrane when growing on a carbohydrate-rich medium. He found that these membranes were soluble in ammoniacal copper hydroxide and gave reducing sugars when hydrolysed with sulphuric acid. Since he knew that cotton gave these reactions he called the organism *Bacterium xylinum* (following PLINY who used the word *xylinum* for cotton). Later investigations by VAN WISSELINGH<sup>3</sup> and HIBBERT<sup>4</sup> demonstrated that the material of these membranes is indeed cellulose. HIBBERT found that membrane is produced only in a culture medium containing hexose sugars, their anhydrides or substances which the organism can readily convert into hexoses. MARK AND VON SUSICH<sup>5</sup> confirmed the identity of bacterial cellulose with cotton cellulose by producing well-oriented x-ray diagrams from stretched bacterial membranes that were the same as those from cotton. The original electron micrographs of FRANZ AND SCHIEBOLD<sup>6</sup> showed that these bacterial membranes consist of a thick network of fibres and ribbons. They concluded that the ribbons had a thickness of ca 100 Å, a breadth of ca 5000 Å and that they were composed of smaller fibres of ca 200 Å diameter. The fact that the cellulose fibrils in the walls of plant cells have nearly the same size now raises the question of whether these are indeed primary structural units of cellulose itself. The present paper describes results obtained in attempts to examine this question using electron micrographs of bacterial cellulose.

## EXPERIMENTAL

Our previous work has shown (FREY-WYSSLING AND MÜHLETHALER<sup>7</sup>; MÜHLETHALER<sup>8</sup>) that liquid media are best for making electron microscopic preparations of

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bacterial cellulose. We have accordingly grown *Bacterium xylinum* for this purpose in WATERMAN's sucrosebeer solution. This is prepared by adding 40 grams of sucrose to a liter of beer. A portion of such a medium was inoculated by dropping into it a small piece of membrane from an old culture. After a short time slimy fibres extended from this inoculum towards the surface. When they reached it they spread as a fine water-clear skin which quickly thickened and after a few days was too tough to be torn or cut without altering its natural texture. To make preparations for electron microscopy the thin, early film was lifted from the culture medium with a glass microscope slide, washed several times in water and dried, still on the slide. Pseudoreplicas like those used for studying the growth of bacteriophage on an agar surface (EDWARDS AND WYCKOFF<sup>9</sup>) have been made of these. This has been done by pouring collodion diluted with amyl acetate over the surface and draining until dry. The resulting film was floated off onto a water surface, cut into pieces, mounted on grids in the usual fashion and shadowed with either chromium or palladium. It should be emphasized that though these preparations are made as if they were replicas they do not replicate the cellulose film which remains embedded in the collodion and is itself observed in the microscope. In some instances a different kind of preparation was made for microscopy by removing a bit of the bacterial colony with a platinum needle, suspending it in water, drying a droplet of this suspension on a collodion covered screen and shadowing.

#### RESULTS

The young rapidly growing bacteria have characteristic shapes as seen in the electron microscope. They are 2–4  $\mu$  long and are flattened on one end (Fig. 1). Midportions of the bacteria have been coated with an amorphous homogeneous substance which increased with bacterial age and eventually embedded the organisms (Fig. 2). Cellulose fibres have begun to form after this has happened. They appeared first as short-fibred thickenings distributed here and there in the amorphous material (Fig. 3). Long fibres have rapidly grown from these thickenings, seemingly at the expense of the capsular substance. As Fig. 3 indicates this development of cellulose has not taken place within a bacterium or from its surface, but rather at some distance from the organism. This suggests that cellulose results from a sort of enzymatically induced polymerization of the embedding slime that can accordingly be thought of as a kind of cellulose-precursor.

As can be seen from Fig. 4 the cellulose fibres have had from the very first a diameter of ca 250 Å. The individual strands were separated from one another by the amorphous substance which decreased as the cellulose developed and had nearly vanished from older colonies (Figs 5 and 6). The separate fibrils have intertwined to form tangled masses, and in this way built tough membranes which could be torn apart only with difficulty. Where the slimy substance has been lacking, several individual fibres have often been seen lying side by side (Fig. 7) to give the bands already described by FRANZ AND SCHIEBOLD. These bands have commonly been twisted about one another into rope-like structures, but it could not be determined with certainty whether this expressed the original arrangement or was caused by the drying. The piece of membrane shown in Fig. 8 shows this phenomenon especially well. Transverse band structures resembling those observed by FRANZ AND SCHIEBOLD are also discernible here, but it is probable that they are the consequence of mechanical damage.



Fig. 1. Individual bacteria from a young colony of *Acetobacterium xylinum*. Slime has begun to precipitate upon the bacteria. Magnification  $\approx 20000 \times$

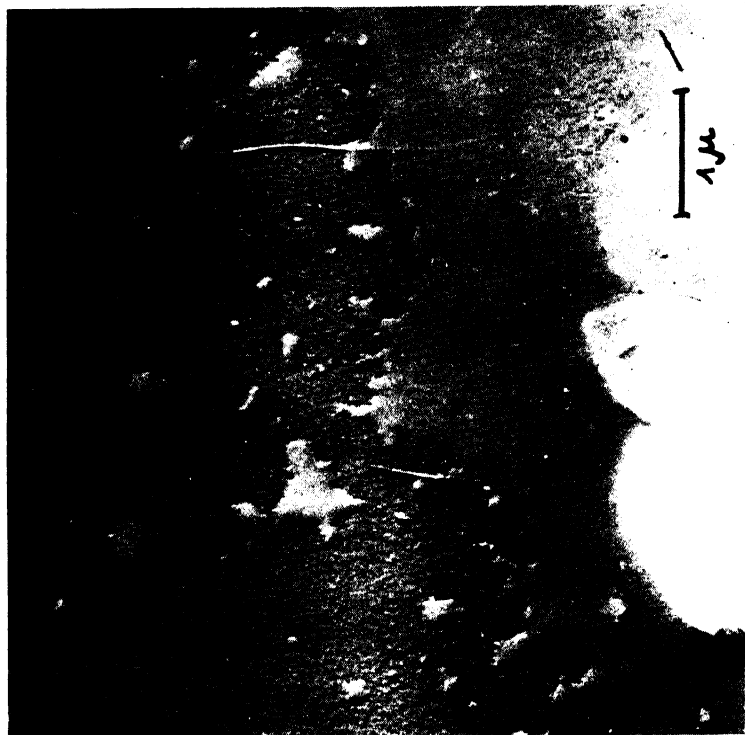


Fig. 2. At a later stage of growth the bacteria are embedded in slime which is still devoid of structure. This photograph shows slime about the edge of a micro colony. Magnification = 18000  $\times$ .

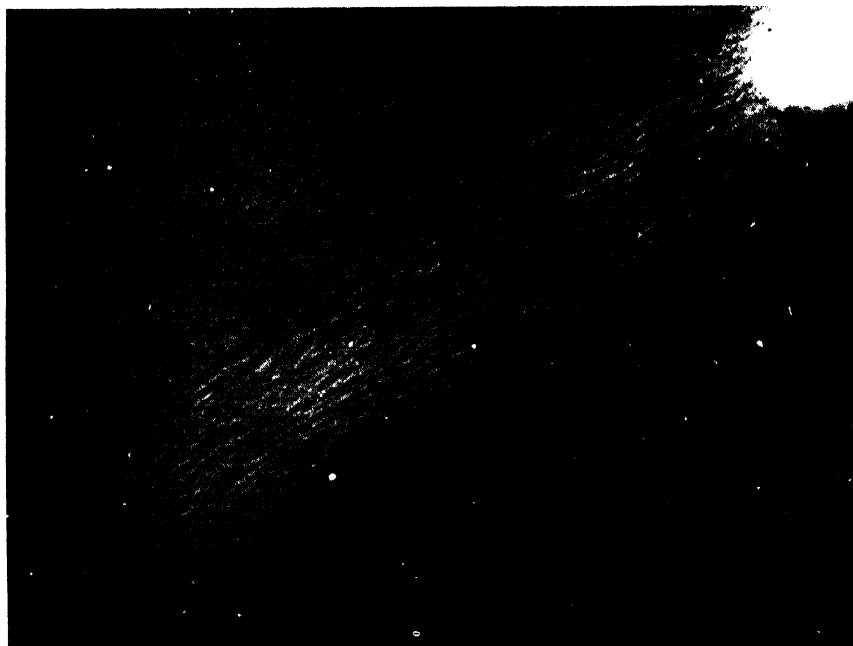


Fig. 3. Strands of cellulose can here be seen forming within the slime. Magnification = 18000  $\times$ .

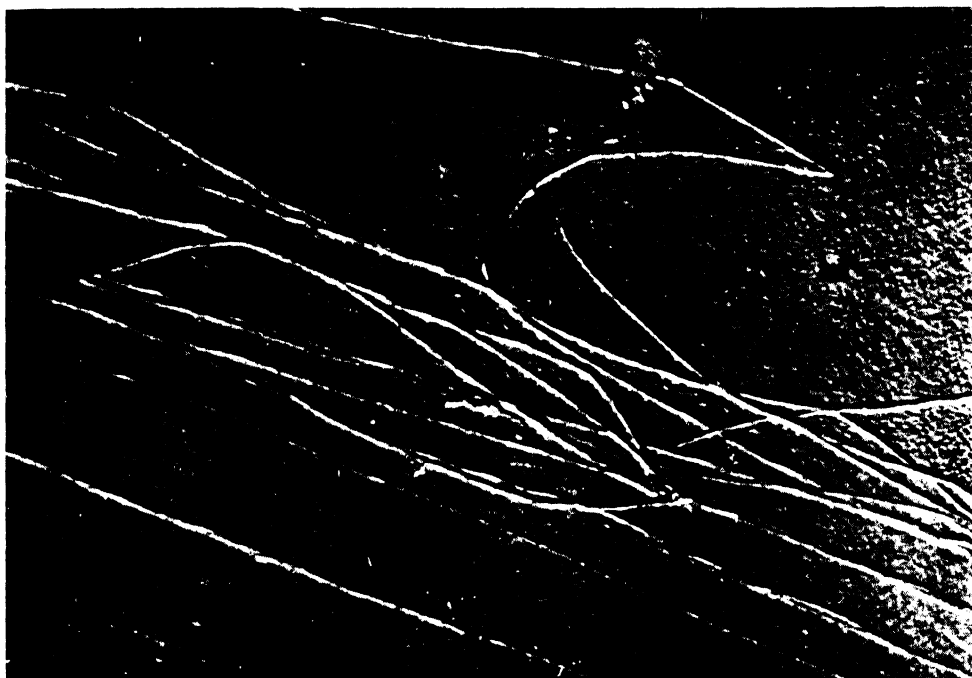


Fig. 4. Several formed cellulose fibrils still embedded in a thin layer of amorphous slime.  
Magnification = 22,000 $\times$ .

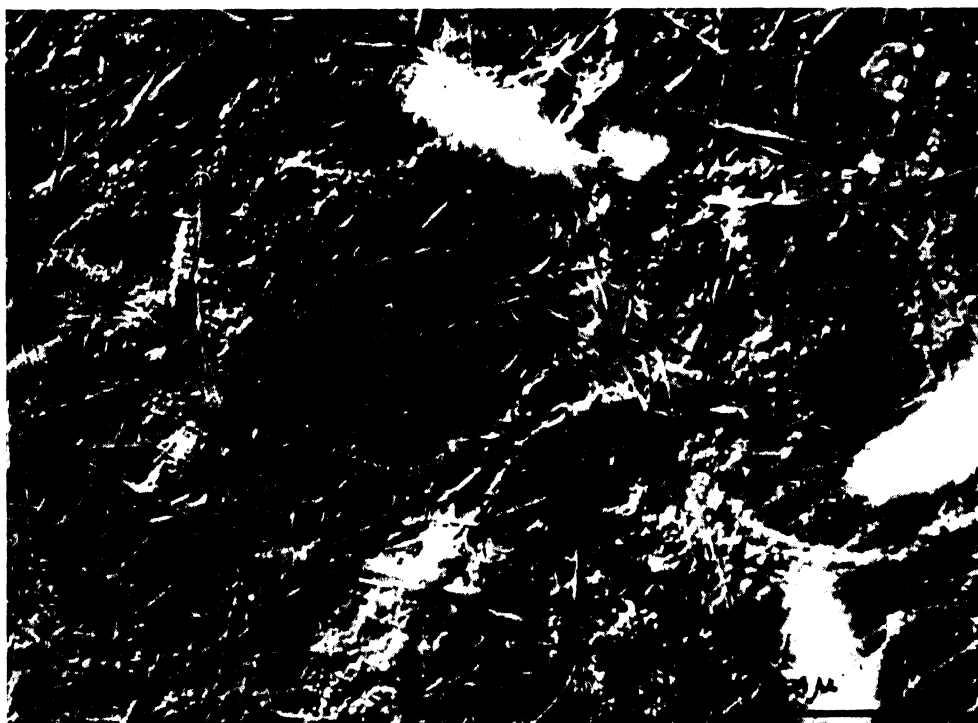


Fig. 5. A dense network of cellulose fibrils. The amorphous stuff has for the most part disappeared.



Fig. 6. Tangled cellulose fibrils in an old culture. Magnification = 22000  $\times$



Fig. 7. Long bands formed by the parallel association of several cellulose fibrils.  
Magnification = 20000 X





Fig. 8. A network of cellulose containing many twisted bands of fibres. Magnification = 22000×

This work was carried out in the laboratory of Dr RALPH W. G. WYCKOFF, whom I wish to thank for many helpful discussions.

### SUMMARY

Electron microscopic observations show that the cellulose built by *Acetobacterium xylinum* occurs in the form of fibres having about the same diameter (ca 250 Å) as that of the fibrils of cellulose from the cell walls of numerous plants. In *Bacterium xylinum* these strands are synthesized outside the cells and can therefore be examined especially well under the electron microscope.

The bacteria first secrete a structurally homogeneous slimy substance within which, after a short time, the cellulose fibres can be seen forming. This secreted substance appears to be a precursor of cellulose whose polymerization proceeds outside the organisms presumably with the help of an enzyme. In older cultures the cellulose strands have become so intertwined as to build a rigid framework.

### RÉSUMÉ

Les observations faites au microscope électronique montrent que la cellulose synthétisée par *Bacterium xylinum* se présente sous forme de fibres ayant à peu près le même diamètre (environ 250 Å) que celui des fibrilles de cellulose constituant les parois cellulaires de nombreux végétaux. Chez *Bacterium xylinum*, ces fibres sont synthétisées à l'extérieur des cellules et peuvent par conséquent être examinées particulièrement facilement au microscope électronique.

Les bactéries secrètent d'abord une substance mucilagineuse homogène, au sein de laquelle on peut voir se former après un temps court, les fibres de cellulose. La substance ainsi sécrétée est un précurseur de la cellulose dont la polymérisation se fait à l'extérieur des organismes, et probablement sous l'action d'une enzyme. Dans les cultures âgées, les fibres de cellulose sont tellement entrelacées qu'elles constituent un entrelac rigide.

### ZUSAMMENFASSUNG

Beobachtungen mit Hilfe des Elektronenmikroskops lassen erkennen, dass die durch *Bacterium xylinum* synthetisierte Cellulose aus Fasern besteht, die ungefähr den gleichen Durchmesser haben (ca. 250 Å) wie die Fasern der Zellwände vieler Pflanzen. Bei *Bacterium xylinum* werden diese Fasern ausserhalb der Zelle synthetisiert und können deshalb besonders leicht im Elektronenmikroskop beobachtet werden.

Die Bakterien scheiden zuerst eine schleimige homogene Substanz aus und nach kurzer Zeit kann man sehen, wie sich innerhalb dieser Substanz die Cellulosefasern bilden. Diese Substanz ist ein Vorläufer der Cellulose deren Polymerisation sich ausserhalb der Organismen, wahrscheinlich unter dem Einfluss eines Enzyms vollzieht. In älteren Kulturen sind die Cellulosefasern so verzweigt, dass sie ein steifes Netzwerk bilden.

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## EFFET DE QUELQUES DÉTERGENTS SUR L'ŒUF DE *TEREDO NORVEGICA*

par

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### I. MATÉRIEL ET TECHNIQUE

L'œuf de *Teredo norvegica* répond à l'action de quelques détergents d'une manière tout à la fois caractéristique, rapide et nuancée, qui fait de lui un matériel cellulaire particulièrement favorable à l'expérimentation.

Les Tarrets utilisés proviennent de bois d'épaves recueillis dans la baie de Concarneau et conservés dans les viviers du laboratoire. Ces bois sont attaqués au ciseau, et les galeries sont progressivement et soigneusement découvertes; puis les individus en sont extraits avec précaution, rapidement lavés dans de l'eau de mer chaque fois renouvelée, et placés séparément dans des boîtes de Petri avec une petite quantité d'eau de mer. Ils peuvent être conservés de la sorte à la température de 3° à 5° C pendant plus de douze heures sans inconvénient.

La ponte des œufs, comme l'éjaculation du sperme, se produit spontanément après l'isolement des individus *in vitro*; l'état des produits sexuels est vérifié, dans les boîtes de Petri, sous une loupe binoculaire. Une ponte étant choisie, les oocytes sont séparés par décantation, lavés et réunis dans un petit cristalliseur. Leur fécondation est effectuée, au moment voulu, en les mélangeant avec du sperme, dont l'excédent est éliminé, quelques minutes plus tard, par décantation et lavage. Le premier globule polaire apparaît 25 à 30 minutes après la fécondation et la première division de segmentation est réalisée environ une heure plus tard.

Les essais comparatifs sont effectués de la manière suivante: les œufs sont prélevés à un stade déterminé et distribués en quantités petites et sensiblement égales, dans une série de petits cristalliseurs en verre pyrex contenant déjà un mélange d'eau de mer, et d'une solution de détergent dans l'eau de mer, de titre connu; les volumes sont calculés de telle sorte que leur somme soit toujours égale à 5 ml, et que toutes autres conditions étant égales par ailleurs, les concentrations des corps étudiés s'échelonnent entre les limites choisies. Les observations sont facilement poursuivies dans les mêmes cristalliseurs, sous une loupe binoculaire; elles sont éventuellement complétées par le prélèvement de petites quantités d'œufs qui sont examinées sous un fort objectif, ou bien fixées et montées après coloration convenable en préparations permanentes.

Les détergents utilisés se répartissent de la manière suivante\*:

#### 1. Détergents anioniques:

Duponols ME, G, D, SO (alcoyle-sulfates).

Igepon T (Sulfate de Na de l'oléylméthyltaurine).

Ucenol LS (Sulfate double de lauryle et de sodium).

Ucenol OCS (Sulfate double d'oléocétyle et de sodium).

Avirol AH (Sulforicinoléate d'alcoyle).

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### 2. *Détergents cationiques:*

Sapamine A (acétate de Diéthylaminoéthyloléylamide).

### 3. *Détergents neutres:*

Cemulsol B (complexe d'acide ricinoléique et d'oxyde d'éthylène polymérisé).

Tween 80 (sorbitan-monooléate et oxyde d'éthylène polymérisé).

Lorsque ces détergents sont utilisés dans les conditions précitées, c'est-à-dire dans l'eau de mer, les composés anioniques se montrent seuls actifs.

L'effet produit par les détergents actifs se manifeste d'une part sur la membrane vitelline de l'œuf, qui peut être gonflée ou dispersée, et de l'autre sur la surface protoplasmique proprement dite; il se traduit alors soit par quelques anomalies de la segmentation, soit par des phénomènes cytolytiques tels que l'exsudation de lobes hyalins, ou la destruction du cortex et la dispersion du contenu protoplasmique.

## II. CONCENTRATION EFFECTIVE DES DÉTERGENTS ANIONIQUES

La préparation d'une solution de détergent de concentration exactement connue est au moins difficile, pour la double raison que les produits commerciaux utilisés ne sont pas nécessairement des corps purs, et que certains d'entre eux, faiblement solubles, se dispersent dans l'eau en suspensions stables, le taux de substance réellement dissoute restant indéterminé. Il apparaît cependant que, à des taux supposés semblables, les solutions des divers détergents anioniques utilisés: Duponol D, G, ME, Igepon T, etc., agissent de manière sensiblement analogue\*. Les concentrations utilisables avec l'œuf de *Teredo* sont de l'ordre de 0.1 à 0.0002%, soit environ  $2 \text{ M } 10^{-4}$  à  $5 \text{ M } 10^{-6}$  par litre.

Aux concentrations de 0.1 à 0.01%, les détergents anioniques provoquent très rapidement une cytolyse totale de l'œuf, comportant la destruction de la membrane vitelline et la dispersion de toute la masse protoplasmique; ou tout au moins, l'aspect caractéristique de la "cytolyse noire", c'est-à-dire la transformation de l'œuf en une masse granuleuse plus ou moins fortement gonflée. On sait que, pour ces mêmes concentrations, divers expérimentateurs ont obtenu des effets bactériostatique et hémolytique (voir DUBOS<sup>1</sup>, PUTNAM<sup>2</sup>).

Aux concentrations de 0.005 à 0.0005%, les mêmes détergents agissent d'une manière beaucoup plus ménagée, et beaucoup mieux adaptée aux possibilités d'une analyse expérimentale.

La concentration de 0.0005% approche de la limite, ses effets sont souvent à peine sensibles; la segmentation des œufs se poursuit et donne une plus ou moins forte proportion de larves apparemment normales.

La concentration de 0.00025% est généralement sans effet.

Utilisés dans les mêmes conditions et aux mêmes concentrations, les détergents cationiques tels que la Sapamine, ou neutres tels que le Cemulsol et les Tweens, restent sans effet sur l'œuf de *Teredo*, ou bien n'agissent que très faiblement et d'une manière incertaine.

Comparant aux détergents anioniques un composé cytolytique dont on peut supposer qu'il agit comme un enzyme, tel le venin d'Abeille avec sa lécithinase, on constate que l'effet cytolytique reste brutal jusqu'à la concentration de 0.005%, qu'il est

\* Le Duponol SO est, cependant, beaucoup moins actif.

ménagé pour 0.0005% environ, et sensiblement nul pour 0.0002%; l'œuf de *Teredo* est donc plus sensible au venin d'Abeille qu'aux détergents anioniques, mais les concentrations limites restent de même ordre dans les deux cas.

L'œuf d'un autre Invertébré marin du type Spiralia, celui de *Sabellaria alveolata*, a été utilisé comparativement; il apparaît aussitôt que cet œuf, dont la taille n'est que de peu supérieure à celle de l'œuf de *Teredo norvegica*, est environ 20 fois moins sensible que ce dernier à l'action des détergents anioniques et 200 fois moins à celle du venin d'Abeille. Dans les deux cas, en effet, les solutions à 0.005% sont inactives, et les manifestations caractéristiques d'une action cytolytique ménagée n'apparaissent qu'aux concentrations de 0.1 à 0.01%.

### III. EFFET SUR LA MEMBRANE VITELLINE

L'action des détergents anioniques sur la membrane vitelline est d'abord marquée par un gonflement qui augmente sensiblement sa surface et provoque son écartement du cortex ovulaire; en présence d'Igepon, cet état persiste le plus souvent (Fig. 4). Dans tous les cas, cependant, la membrane gonflée est très fragile, de sorte qu'une simple agitation mécanique suffit à la rompre, ou même à la détruire.

Si l'attaque est plus marquée, sous l'effet des Duponols, par exemple (Fig. 1), le gonflement s'accroît tout d'abord ainsi que le plissement de la membrane qui forme autour de l'œuf une enveloppe chiffonnée, avant de se disperser en une poussière de

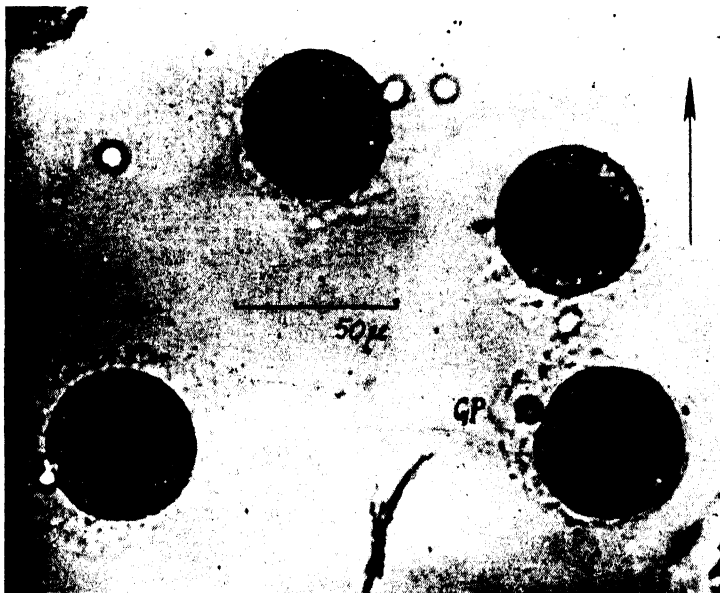


Fig. 1. Œuf de *Teredo norvegica*, avant la première division; la membrane vitelline est gonflée et fortement plissée sous l'action du Duponol G.

granules. Une telle dispersion se produit immédiatement si l'on transporte les œufs dans l'eau de mer pure, ou mieux encore dans une solution isotonique de NaCl.

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Le venin d'Abeille agit d'une manière différente; la membrane se gonfle très peu, mais se rompt en un point, tandis que le bord libre se replie sur lui-même vers l'intérieur; il suffit alors d'une légère agitation pour séparer et isoler ces membranes; elles persistent telles quelles et peuvent être lavées, séchées sur lame de verre et colorées.

La sensibilité ou la fragilité de la membrane vitelline s'accroît légèrement au cours de la maturation et jusqu'à la première division de segmentation, c'est-à-dire durant la période correspondant à son écartement normal du cortex oculaire.

#### IV. EFFET SUR LE CORTEX OVULAIRE

La membrane vitelline paraît, quel que soit son état d'altération, demeurer perméable aux détergents utilisés, car ceux-ci exercent une action cytolytique très manifeste sur le cortex ovulaire; ce terme de *cortex* sera utilisé ici, car il ne préjuge ni de la nature, ni du degré de différenciation de la couche cellulaire superficielle correspondant à l'interface cytoplasma-milieu liquide extérieur.

Les détergents anioniques utilisés aux concentrations supérieures à 0.005% provoquent une altération corticale accompagnée par un gonflement de la masse cytoplasmique et l'apparition de hernies granuleuses; aux concentrations voisines de 0.01%, ces hernies peuvent devenir très importantes et très fragiles; leur rupture entraîne la dispersion de tout le matériel cytoplasmique.

Aux concentrations inférieures à 0.01%, l'effet cytolytique se traduit par la formation de lobes hyalins réfringents comparables aux "boules sarcodiques" si souvent décrites au cours de l'altération d'œufs et de Protozoaires et caractéristiques de la "cytolysé claire".

Enfin, lorsque la concentration des détergents anioniques est proche de sa valeur limite, l'altération corticale provoquée ne se manifeste plus guère que par une forte tendance à l'isolement des premiers blastomères, qui, après la division, restent sphériques au lieu de s'étaler secondairement l'un sur l'autre. Leur simple contact tangentiel n'est plus, dès lors, qu'un lien très fragile, aisément rompu soit par le gonflement de la membrane vitelline (Figs. 2 et 3), soit par la moindre agitation. La segmentation peut se poursuivre, dans ces conditions, aussi bien sous la membrane distendue que, après la disparition de celle-ci, dans le milieu liquide (Figs 3 et 5); elle peut être qualifiée de *non-cohérente* ou d'*arborescente*, car les divers blastomères ne gardent entre eux, après chaque division, qu'un seul point de contact tangentiel (Fig. 4). Au lieu de rester ramassées en une masse sphérique, les cellules de segmentation peuvent alors s'étaler dans un plan, en chaînes dont les ramifications dichotomiques retracent la "cell-lineage" (Fig. 5).

L'effet cortical des détergents se traduit d'autre part, lorsque l'action cytolytique proprement dite est faible ou nulle, par une forte tendance à l'isométrie des deux premiers blastomères (Fig. 2), contrastant avec la différence de taille accentuée qui, dans la segmentation normale, distingue à première vue les deux cellules AB et CD. Cet effet n'est aucunement spécifique; PENNERS<sup>3</sup>, <sup>4</sup>, TYLER<sup>5</sup>, PASTEELS<sup>6</sup>, l'ont obtenu avec les œufs de différents Spiralia, sous des influences très diverses; on peut le réliaser d'autre part en traitant les œufs de *Teredo* par un mélange d'eau de mer et d'une solution isotonique de KCl, ou bien en les soumettant quelques minutes au rayonnement ultra-violet d'une lampe à vapeur de mercure.

On sait que, selon PENNERS<sup>3</sup>, <sup>4</sup> et selon TYLER<sup>5</sup>, l'égalaion de la première division

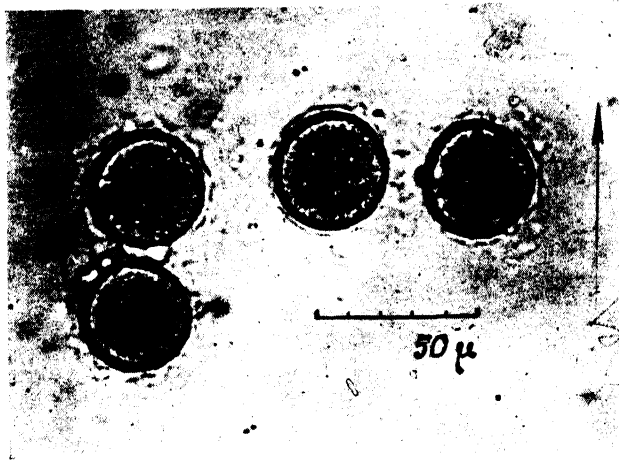


Fig. 2. Première division égale avec séparation complète des deux premiers blastomères. Duponol G

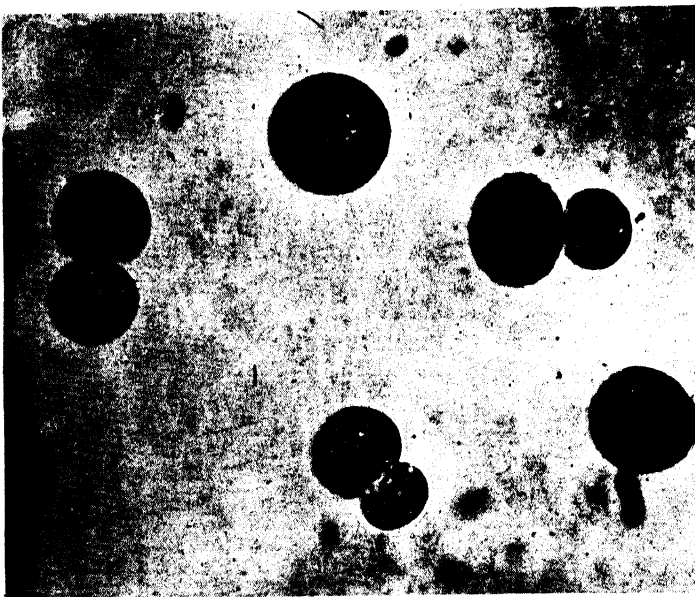


Fig. 3. Deuxième division égale ou inégale des blastomères CD isolés

est supposée correspondre à la formation de deux blastomères CD équivalents, ce qui conduit à la réalisation d'embryons doubles; en fait, chez *Teredo*, la seconde division est généralement anisométrique et correspondrait à la séparation de deux blastomères C et de deux blastomères D. Mais il peut arriver que la seconde division soit encore isométrique, chaque quadrant étant égal aux autres et correspondant encore à un blastomère CD.

L'action ménagée des détergents sur l'œuf de *Teredo* permet encore de mettre en évidence, au cours de la segmentation, un effet de sensibilité différentielle sur lequel

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Fig. 4. Segmentation non-cohérente sous la membrane vitelline persistante mais dilatée. Igepon T



Fig. 5. Segmentation non-cohérente du type arborescent. Duponol ME

nous aurons à revenir. Lorsque cette action s'exerce au moment de la première division ou peu après, on peut obtenir, dans une marge assez étroitement limitée de concentration du corps actif, la cytolysse du gros blastomère CD, alors que AB reste apparemment intact et donne, par sa segmentation ultérieure, une petite morula, puis une petite larve

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ciliée. Le venin d'Abeille à certaines concentrations, provoque le même phénomène, plus nettement encore, peut-être.

En d'autres cas, la sensibilité différencielle se manifeste seulement par le fait que le blastomère CD reste indivis, bien que son noyau se soit multiplié, tandis que AB se segmente et forme une petite masse blastuléenne.

La possibilité d'égaliser la première division de l'œuf de Taret; celle de séparer, d'isoler les premiers blastomères; ou encore d'éliminer ou de bloquer le développement ultérieur de l'un d'entre eux, montrent que les détergents anioniques peuvent être utilisés en technique d'embryologie expérimentale, avec certains œufs tout au moins. Ceci suppose l'emploi de ces corps sous des conditions assez strictement définies pour que soient évitées les cytolyses tardives possibles, et pour que la non-cohérence des cellules reste limitée aux premiers stades de la segmentation et puisse être corrigée ultérieurement. Nombre de faits observés, sur lesquels nous ne saurions insister ici, montrent qu'un tel programme est réalisable.

On remarquera encore qu'après disparition de la membrane vitelline, les blastomères, ou les amas de blastomères provenant de différents œufs, tendent à s'agglutiner, même s'ils ne gardent entre eux que les contacts tangentiels caractéristiques de la segmentation non-cohérente. On obtient ainsi des ensembles plus ou moins volumineux et irréguliers, capables de se condenser ultérieurement et de former des larves monstrueuses multiples comparables à celles réalisées déjà par quelques auteurs, HATT<sup>7</sup>, par exemple, avec des œufs de *Sabellaria* temporairement alcalinisés.

#### V. CONDITIONS D'ACTIVITÉ POUR UN DÉTERGENT CATIONIQUE

Un détergent cationique tel que la Sapamine est, nous l'avons dit, pratiquement sans effet, sur l'œuf de *Teredo*, lorsqu'il est dissous dans l'eau de mer normale, c'est-à-dire dans une solution saline tamponnée, de  $p_H$  8.2; considérant ce fait, nous avons modifié les conditions de nos essais de manière à faire agir la Sapamine en solutions dont le  $p_H$  soit déplacé vers la neutralité, puis du côté acide.

A cet effet, différents mélanges d'eau de mer normale et d'eau de mer acidifiée par HCl sont utilisés; comme dans les précédents essais, le volume liquide total est toujours, après l'addition des œufs, de 5 ml; la Sapamine est au taux constant de 0.01 %; seule la valeur de  $p_H$ , vérifiée colorimétriquement, est différente dans chacun des récipients utilisés.

L'effet propre exercé sur les œufs de *Teredo* par la concentration en ions H de l'eau de mer étant examinée au cours d'expériences témoins, il apparaît que la segmentation s'effectue normalement, jusqu'aux stades larvaires, entre  $p_H$  8.2 et  $p_H$  5.5 l'acidification provoquant, tout au plus, quelques variations de vitesse qui n'ont pas été précisées. Au-dessous de  $p_H$  5.5, par contre, la segmentation est considérablement ralentie ou bloquée, en même temps que les œufs montrent quelques altérations du type "cytolysse noire".

En présence de Sapamine à 0.01 %, la segmentation s'effectue normalement entre  $p_H$  8.2 et  $p_H$  7.0; vers  $p_H$  6.8 on observe déjà, cependant, quelques cas de cytolysse claire. Puis, à  $p_H$  6.2 environ, la première division manifeste une forte tendance à l'égalisation (Fig. 6), tandis que de nombreux aspects cytolytiques, caractérisés par la formation de lobes hyalins, apparaissent au cours des divisions suivantes.

Près de  $p_H$  5.8 l'effet maximum est obtenu; la membrane vitelline se gonfle, se distend, se rompt; les cytolyses claires sont très nombreuses et peuvent aller jusqu'à la destruction de l'œuf. Mais les œufs les moins sévèrement atteints subissent, sous leur membrane distendue, une segmentation non cohérente (Figs 6 et 7), étroitement comparable à celle que provoquent, dans l'eau de mer normale à  $p_H$  8.2, les détergents anioniques. Au-dessous de  $p_H$  5.8 on trouve bientôt la zone d'action propre de l'acidité et les essais avec les détergents ne seraient plus valables.

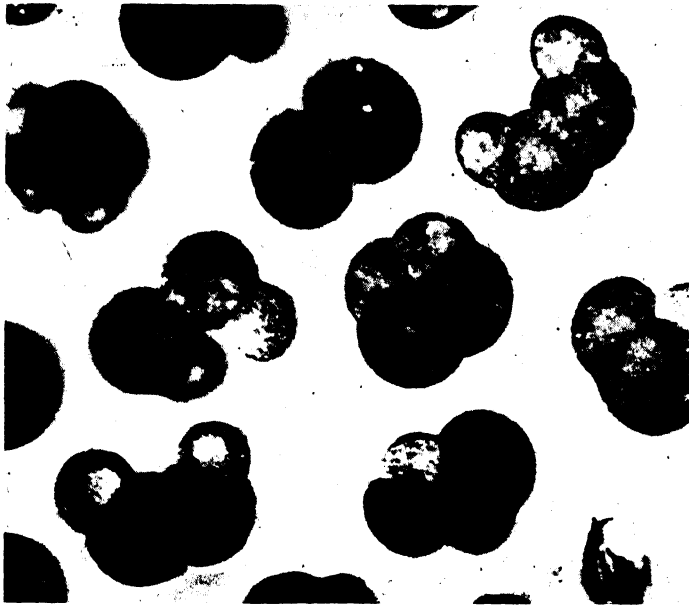


Fig. 6. Stade IV; première division égale ou sub-égale donnant 2 blastomères CD; deuxième division inégale donnant 2 gros blastomères D et 2 petits C, cohérents ou non-cohérents. Sapamine A à pH 6.6



Fig. 7. Segmentation non-cohérente. Sapamine A à pH 6. Comparer avec Fig. 5

Inversement, la même série d'essais réalisés cette fois avec le Duponol ME, montre que l'effet de ce détergent anionique sur l'œuf de Taret est nettement diminué, mais non complètement supprimé, du côté acide, aux environs et immédiatement au-dessous de pH 6.4.

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Il apparaît ainsi que la différence d'action qui distingue si nettement les détergents anioniques et la Sapamine est liée, au moins pour une très large part, à la réaction alcaline ou acide de la solution qui baigne les œufs.

#### VI. INTERPRÉTATION DES RÉSULTATS

Les différents composés appartenant à la catégorie des "détergents" se trouvent apparentés les uns aux autres par une certaine configuration de leur molécule, qui comporte une moitié organique, de caractère non-polaire et hydrophobe, l'autre moitié étant inorganique, de caractère polaire et hydrophile.

NEUBERG<sup>8</sup> a, voici longtemps, attiré l'attention sur le pouvoir émulsifiant et solubilisant de telles molécules à l'égard de substances insolubles ou très peu solubles dans l'eau (voir HÖBER<sup>9</sup>). Considérons l'interface d'une phase organique liquide à molécules non-polaires hydrophobes et d'une phase aqueuse telle qu'une solution d'un détergent ionique, l'affinité d'adsorption des groupes non-polaires provoquera la fixation du groupe organique hydrophobe des molécules du détergent sur le groupe similaire des molécules de la phase organique insoluble; par contre, l'affinité pour l'eau du groupe inorganique polaire des molécules du détergent maintiendra ce groupe dans la phase aqueuse où il aura tendance à diffuser; sa mobilité se transmettra par l'intermédiaire de la chaîne grasse, longue et souple, qui unit les deux groupes, jusqu'au point de fixation sur la surface organique hydrophobe provoquant la dislocation et la dispersion de celle-ci dans la phase aqueuse.

Mais les détergents de structure polaire-non polaire réagissent avec les protéines de manières diverses, et les recherches de KUHN ET BIELIG<sup>10</sup>, de PUTNAM ET NEURATH<sup>11</sup>, de LUNDGREN<sup>12, 13</sup> etc., suggèrent que les liaisons sont alors de nature électrostatique; que des combinaisons apparemment stoechiométriques s'effectuent par le moyen des groupes polaires; qu'elles réalisent ainsi certains complexes mis en évidence par électrophorèse (voir VALKO ET<sup>14</sup> PUTNAM<sup>2</sup>).

On sait que, suivant la concentration utilisée, les détergents peuvent: a) précipiter ou b) disperser les protéines.

a) La précipitation est due à la neutralisation des charges; on constate, par exemple, qu'un détergent anionique précipite une protéine cationique, c'est-à-dire en solution à un  $p_H$  inférieur à son point isoélectrique et *vice versa*. Dans ce cas, les groupes polaires de signe opposé sont en contact et les chaînes organiques des molécules du détergent forment autour de la protéine une enveloppe hydrophobe dont la stabilité est assurée par l'affinité d'adsorption des groupes non polaires tournés vers la phase aqueuse.

b) La dispersion peut se produire ultérieurement si d'autres molécules du détergent peuvent former une seconde couche dont les groupes polaires ionisés sont tournés cette fois vers la phase aqueuse, le contact avec la première couche étant assuré par les groupes organiques non-polaires. On retrouve alors, mais sous une forme plus compliquée, la condition envisagée par NEUBERG et par HÖBER.

Les deux schémas différents et complémentaires résumés ci-dessus doivent être pris en considération à propos de l'action des détergents sur l'œuf de *Teredo*; comme en ce qui concerne leurs effets sur les Bactéries (voir PUTNAM<sup>2</sup>).

On remarquera d'abord, avec PUTNAM<sup>2</sup>, que les détergents ioniques se comportent, aux concentrations voisines de 1/3000 et au-dessous, comme des électrolytes forts, de sorte que les variations de  $p_H$  affectent l'ionisation de la surface cellulaire et non point celle du détergent.

On constatera ensuite que l'on sait très peu de chose en ce qui concerne les constituants du cortex cytoplasmique des œufs de *Spiralia* en général, mais que le signe et la valeur de leur charge superficielle globale peuvent être connus par électrophorèse. Ce dernier point n'a pas été examiné en ce qui concerne l'œuf du Taret, mais les données obtenues pour d'autres œufs peuvent être invoquées à son égard.

On sait en effet que, suspendues en milieu aqueux neutre ou alcalin, la plupart des cellules vivantes accusent, par électrophorèse, une charge superficielle négative (voir, par exemple: WINSLOW, FALK ET CAULFIELD<sup>15</sup>; FAURÉ-FREMIET ET NICHITA<sup>16</sup>; PFEIFFER<sup>17</sup>; ABRAMSON<sup>18</sup>; HÖBER<sup>9</sup>, etc). Lorsque la concentration en ions  $H^+$  augmente dans le milieu liquide, cette charge négative décroît, s'annule, puis devient positive; le point d'inversion correspond souvent à un  $p_H$  trop acide pour rester compatible avec un état normal de la cellule vivante, mais il peut être déplacé vers la neutralité, voire même du côté alcalin, en présence des cations bivalents  $Mg^{++}$ ,  $Ca^{++}$ , ou trivalents:  $La^{+++}$ , par exemple.

En ce qui concerne les œufs d'Animaux marins, KATSUMA DAN<sup>19, 20, 21, 22</sup> a retrouvé les mêmes caractéristiques générales chez ceux de divers Echinodermes, Annélides et Mollusques, ce qui autorise à interpréter le comportement des œufs de *Teredo norvegica* en supposant qu'ils ne font pas exception à la règle, et possèdent dans l'eau de mer à  $p_H$  8.2 une charge superficielle négative. Nous ne discuterons pas l'origine de cette charge, qui peut être liée soit à l'existence de groupements ionisés négatifs (zwitterions protéiques, par exemple) soit aux propriétés de l'eau et à l'orientation de ses dipôles (comme dans le cas de particules neutres, gouttes de carbures ou bulles d'air, p. ex.).

Nous admettrons dès lors, en première approximation, que la charge superficielle négative de l'œuf repousse les groupes polaires de même signe portés par les molécules de détergents anioniques, et que, de ce fait, les chances d'union entre les groupes non-polaires appartenant aux molécules du détergent et à certains constituants de la surface protoplasmique se trouvent augmentées; l'œuf est donc entouré par une couche de molécules dont les groupes hydrophiles, en contact avec le liquide extérieur, exercent leur action solubilisante conformément au schéma proposé par NEUBERG et par HÖBER. Cette action se traduit par l'altération du cortex ovulaire et par la cytolypse.

Inversement, cette même charge négative de l'œuf attire les groupes polaires de signe opposé portés par les molécules des détergents cationiques; celles-ci s'orientent avec le groupe hydrophobe non-polaire à l'extérieur et constituent ainsi autour de l'œuf une couche protectrice.

Si cette hypothèse est valable, une diminution et une inversion de la charge superficielle de l'œuf devra progressivement diminuer les chances d'un tel mode d'association entre les molécules du détergent et celles du cortex ovulaire, puis renverser le sens de cette association. C'est précisément ce que l'on observe avec l'œuf de *Teredo*, entre  $p_H$  6.8 et  $p_H$  5.5, au-delà duquel l'effet propre de l'acidité interdit de poursuivre les essais.

L'œuf de *Teredo norvegica* apparaissant, répétons-le, comme un matériel particulièrement sensible à l'action des détergents synthétiques, il est utile de confronter les indications qu'il apporte avec celles fournies par d'autres cellules vivantes. Il apparaît, et il est nécessaire d'insister sur ce point, que l'effet apparent des mêmes catégories de détergents peut varier dans une large mesure, suivant que ces corps agissent sur telle sorte de cellule ou sur telle autre.

MONNÉ<sup>23</sup>, comme BAUD<sup>24</sup>, obtiennent en milieu salin neutre, avec des détergents anioniques agissant aux concentrations de 1.0 à 0.50% sur des cellules de Mollusques

et de Mammifères, un effet dispersif conduisant à la cytolyse totale. Ce résultat est identique à celui obtenu avec l'œuf de Taret, et s'accorde avec la notion que les molécules actives se fixent sur le cortex cellulaire (chargé négativement) par le jeu d'affinités de cohésion entre groupes non-polaires.

Par contre, les détergents cationiques utilisés dans les mêmes conditions provoquent sur les mêmes cellules des effets particuliers de précipitation, que l'on peut rapporter à des constituants protéiques corticaux, et qui, rappelons-le, n'apparaissent pas avec l'œuf de Taret. Si donc on est en droit d'admettre ici l'interaction des groupes polaires, on doit remarquer que les conséquences de la fixation électrostatique d'une couche de molécules de détergent sur le cortex de la cellule peuvent différer considérablement d'un cas à un autre.

Chez les Bactéries, il semble que les effets typiquement lytiques soient, lorsqu'ils apparaissent, de nature secondaire; et que l'action immédiate des détergents se manifeste d'une toute autre manière par un effet bactériostatique, lié au blocage des oxydo-réductions. Il en est ainsi pour toutes les Bactéries, avec les détergents cationiques en milieu alcalin; et pour les Bactéries Gram-négatives, avec les détergents anioniques en milieu acide. Dans l'un et l'autre cas la liaison électrostatique des molécules actives et du cortex cellulaire est au moins probable. Avec les Bactéries Gram-positives, cependant, le même effet bactériostatique est provoqué par les détergents anioniques en milieu neutre ou alcalin, c'est-à-dire dans les conditions qui provoquent la lyse de diverses cellules animales et de l'œuf de Taret, et pour lesquelles on peut admettre

la fixation des molécules actives par affinité d'adsorption des groupes non-polaires.

On voit ainsi, par cette diversité d'effet, que les détergents ou, d'une manière générale, les corps à structure polaire — non-polaire, peuvent fournir à la cytologie expérimentale et à l'étude du cortex cellulaire, un précieux moyen d'analyse, si, toutefois, quelques informations complémentaires peuvent être parallèlement demandées à d'autres moyens, comme MONNÉ<sup>23</sup> et comme BAUD<sup>24</sup> ont commencé de le faire.

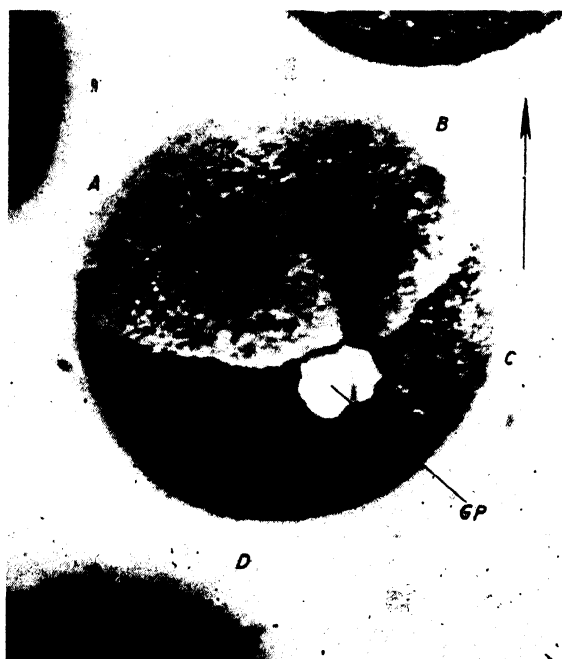


Fig. 8. Œuf de *Taredo norvegica* normal imprégné à l'argent; Stade IV vu par le pôle animal; GP.: trace de deux globules polaires; le blastomère D est fortement coloré, le blastomère C est moins décoloré que A et B (E. FAURÉ-FREMIET ET H. MUGARD).

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## VII. NATURE DU CORTEX OVULAIRE

FAURÉ-FREMIET ET MUGARD<sup>25</sup> montrent que le cortex de l'œuf de Taret présente, dans certaines conditions techniques, une affinité remarquable pour l'Argent; et que cette affinité s'atténue ou disparaît après action de KCl, et, mieux encore, des détergents anioniques

utilisés aux mêmes concentrations qui modifient et altèrent le processus normal de la segmentation.

L'argyrophilie de l'œuf de *Teredo* paraît être liée à la présence d'un matériel cortical labile, et certains résultats laissent penser que celui-ci pourrait être de nature lipoprotéique, bien que cette hypothèse reste à démontrer. Quoiqu'il en soit, on doit constater un certain parallélisme entre l'effet des détergents et la présence de ce matériel.

En effet, FAURÉ-FREMIET ET MUGARD montrent encore que l'argyrophilie corticale se restreint progressivement, au cours de la segmentation inégale de l'œuf de Taret, à la surface des gros blastomères CD, puis D, et (Figs 8 et 9) ultérieurement, à quelques-uns de leurs dérivés, ce qui doit être rapproché de quelques constatations exposées ici, à savoir :

1. que l'un des effets caractéristiques des détergents anioniques, qui diminuent ou suppriment l'argyrophilie, est d'égaliser la première division de l'œuf, c'est-à-dire d'empêcher la différenciation des deux blastomères AB et CD ;

2. que les blastomères les plus sensibles à l'effet de ces détergents sont précisément ceux sur lesquels se localise le matériel argyrophile, soit CD, puis D ;

3. que l'œuf de *Sabellaria*, sensiblement moins argyrophile que celui de *Teredo*, est notablement moins sensible que celui-ci à l'action de ces mêmes corps.

Ces remarques suggèrent qu'il peut exister une relation directe entre la sensibilité de l'œuf de *Teredo* aux détergents anioniques (et à d'autres corps lysants tels que le venin d'Abeille) et la présence du matériel cortical argyrophile.

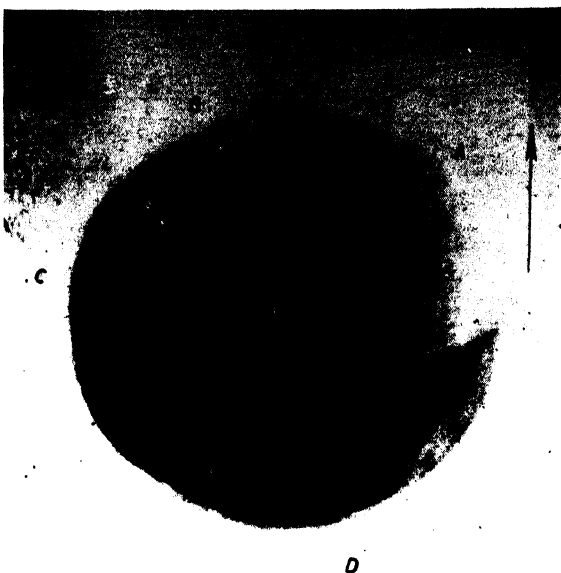


Fig. 9. Œuf de *Teredo norvegica* normal imprégné à l'argent ; Stade IV vu par le pôle végétatif ; mêmes remarques que pour Fig. 8 (E. FAURÉ-FREMIET ET H. MUGARD)

#### RÉSUMÉ

Divers détergents anioniques en solution dans l'eau de mer à pH 8.2 provoquent la cytolysse de l'œuf de *Teredo norvegica*, tandis que des détergents neutres ou cationiques sont sans effet à la concentration de 0.1 %.

La neutralisation et l'acidification de l'eau de mer inversent l'action de ces corps ; au-dessous de pH 6.8 par exemple, un détergent cationique tel que la Sapamine agit comme un détergent anionique en milieu alcalin.

Il est supposé que l'effet lytique se produit lorsque les molécules du corps actif sont fixées sur le cortex ovulaire par affinité entre groupes non-polaires, leur groupe ionisé restant au contact de l'eau environnante ; et que cette condition est remplie lorsque la charge de surface de l'œuf est de même signe que le groupe polaire de la molécule du détergent et repousse celui-ci.

La comparaison avec les résultats obtenus par divers auteurs est en accord avec cette interprétation, compte tenu du fait que la liaison des détergents avec le cytoplasme cortical peut avoir, suivant la cellule considérée, des conséquences différentes.

La sensibilité de l'œuf de *Teredo* aux détergents paraît être liée à la présence, chez cet œuf, d'un constituant cortical de nature probablement lipo-protéique.

## SUMMARY

Several anionic detergents when dissolved in sea water at pH 8.2 cause cytolysis of the egg of *Teredo norvegica*, while neutral or cationic detergents have no effect at a concentration of 0.1 %.

Neutralization and acidification of the sea water reverse the action of these substances. For example, below pH 6.8 a cationic detergent such as Sapamine acts like an anionic one in an alkaline medium.

It is supposed that lysis occurs when the molecules of the active substance are attached to the ovular cortex by affinity between non-polar groups, its ionized groups remaining in contact with the surrounding water. This condition is fulfilled when the surface charge of the egg has the same sign as that of the polar group of the detergent molecule therefore repulsing it.

The comparison of the results obtained by different authors is in agreement with this interpretation, taking into account that the bond between the detergents and the cytoplasm of the cortex may have different results, according to the cell used.

The sensitivity of the egg of *Teredo* to detergents seems to be related to the presence, in that egg, of a cortical constituent, probably a lipo-protein.

## ZUSAMMENFASSUNG

Verschiedene anionische Netzmittel bewirken, in Meerwasser bei pH 8.2 gelöst, die Zytolyse des Eis von *Teredo norvegica*, während neutrale oder kationische Netzmittel bei einer Konzentration von 0.1 % wirkungslos sind. Durch Neutralisieren oder Ansäuern des Meerwassers wird diese Wirkung umgekehrt, indem zum Beispiel unter pH 6.8 ein kationisches Netzmittel wie Sapamin wie ein anionisches in alkalischem Medium wirkt.

Es wird angenommen, dass die auflösende Wirkung erfolgt wenn die Molekeln des aktiven Stoffes an den Cortex des Eis durch die Affinität zwischen nicht-polaren Gruppen gebunden sind, während seine ionisierten Gruppen in Kontakt mit dem umgebenden Wasser bleiben. Dies ist der Fall wenn die Oberflächenladung des Eis dasselbe Vorzeichen hat wie die polare Gruppe der Netzmittelmolekel und diese daher abstößt.

Ein Vergleich der Versuchsergebnisse verschiedener Forscher stimmt mit dieser Auslegung überein, wenn man in Betracht zieht, dass die Bindung zwischen dem Netzmittel und dem Protoplasma der Cortex, je nach Zelle, verschiedene Folgen haben kann.

Die Empfindlichkeit des Eis von *Teredo* gegen Netzmittel scheint mit einem Bestandteil des Cortex, wahrscheinlich einem Lipo-proteid zusammenzuhängen.

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## THE SUBMICROSCOPIC ORGANIZATION OF THE WALLS OF CONIFER CAMBIUM

by

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### INTRODUCTION

The physiological significance of the cambium as the major lateral meristem of arborescent plants has long been recognized and has been the subject of intensive investigation, notably by BAILEY (1923) and by PRIESTLEY (1930). Information regarding the submicroscopic organization of this tissue is, however, meagre and investigation from this point of view in conifers has hitherto centred more on the secondary wall of the xylem tracheids which arise from the cambium by its regular division. The structure of the secondary walls is now known in some detail (BAILEY AND VESTAL, 1937; PRESTON, 1946; WARDROP AND PRESTON, 1947) and it is therefore important to examine the cambium from this point of view.

Differences in structure between the primary walls of the cambial initials and the secondary layers of tracheids are clearly to be expected, since the cambium remains alive throughout the life of the tree and its walls continue to undergo dimension changes, during each growing season, over a period of 30–50 years or more, while the tracheids reach their final size soon after differentiation. Such differences as these may well have an important bearing on the processes of growth.

In attempting to elucidate the structure of the wall in such a growing tissue, it is clearly essential to observe material under conditions as nearly as possible like those obtaining in the living tissue. This is always the major difficulty in attempting observations of a biophysical nature and we cannot in the present instance claim to have overcome it completely. It is possible that certain of the more confusing aspects of our present knowledge of wall structure in growing cells is to some extent attributable to various degrees of success achieved by different workers in preserving the tissues in their natural state.

In problems of submicroscopic structure of growing cells generally it is to be remembered that there are many aspects of molecular organization to be assessed, of which perhaps the most important are:

1. The presence or absence of cellulose, and the nature of other substances present and of their inter-relations.
2. Whether the cellulose possesses micellar organization and, if so, the size of the micelles.

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3. The relative amount of cellulose which is organized into micelles — the so-called crystalline/non-crystalline ratio.

4. The preferred orientation of the micelles, if any.

5. The angular dispersion about the direction of preferred orientation.

On very few of these points has general agreement been reached. Thus BERKELEY AND KERR (1946) claim that in cotton hairs in the fresh condition the molecular chains of cellulose in the wall, while lying more or less parallel to each other as shown by the birefringence, are not organized into micelles since no reflection of X-rays occurs such as one expects from material with crystalline organization. On stretching or drying the cells, however, the cellulose apparently "crystallizes" and gives a cellulose diffraction pattern of the normal type. From these observations BERKELEY AND KERR concluded that in the fresh condition the chains of cellulose are separated by water films probably two molecules thick. On the other hand the same authors could not demonstrate these phenomena in young stems of flax where the X-ray diagram typical of micellar cellulose was obtained under all conditions, and attribute this to the early operation of growth stresses. It is not clear, however, that other factors are not involved and these will be discussed later in the paper. The frequent difficulty found in staining young growing walls with  $I_2$  and  $H_2SO_4$  might also be explicable along these lines, although here again many factors are concerned since difficulty of staining is encountered with other cells in the walls of which micellar cellulose is definitely present (ASTBURY AND PRESTON, 1940). The percentage cellulose in growing walls is known to be low (ALLSOPP AND MISRA, 1940; BONNER, 1935), but beyond the general picture presented by BERKELEY AND KERR nothing is known concerning the crystalline/non-crystalline ratio.

As regards the orientation of the micelles it is agreed that, in a wide variety of elongating cell types, the micelles tend to be in a comparatively flat spiral (VAN ITERSON, 1935; PRESTON, 1947; MAAS GEESTERANUS, 1941; FREY-WYSSLING, 1948) though in some of these cases it is not clear that the observations made are of a type which allows of unambiguous interpretation and in some cases (KUNDU AND PRESTON, 1940; PRESTON, 1941) it is suggested that at least in some stages of elongation the micellar spiral is steep. The most interesting feature of growing cells with a demonstrably flat spiral organization, is that the pitch of the spiral remains unaltered during elongation.

It is against this general background that the present structural determination was attempted on the primary walls of cambial initials in the conifer. Previous investigations have shown that the birefringence of cambial initials is negative (VAN ITERSON, 1935) indicating an angle between the general micelle direction and cell length greater than  $45^\circ$  and that in the primary layer which still surrounds the mature tracheid the corresponding angle is in fact about  $80^\circ$  on the average (PRESTON, 1947). It was thought desirable to establish the condition in the cambium itself by an X-ray method and the present paper deals largely with the result of the consequent investigation.

#### X-RAY AND OPTICAL INVESTIGATION OF CONIFER CAMBIUM

The bulk of the X-ray investigations was made on air dried material and this will be considered first leaving, the work on wet material to be briefly discussed later in this paper. The major difficulty in the X-ray examination of the cambium is to obtain specimens of sufficient thickness to give a good diffraction photograph. This was achieved by peeling the cambium from young specimens of *Pinus sylvestris* early in the growing

season in the manner long used in this laboratory and as described by PRIESTLEY AND MALINS (1933), the material being collected when the cells were in active division and before any secondary wall deposition had occurred. The long delicate strips of tissue, some 3-4 cells thick, were kept in dilute preservative and subsequently prepared for examination by washing in distilled water. A block some 3 mm wide and 10 mm long was built up by laying successive strips over each other in parallel orientation, which were then dried flat on a glass slide. During drying, the cambial cells collapsed and became flattened in the plane of the strips so that in effect the block was a series of superposed parallel walls. The diffraction photographs from specimens thus prepared, however, showed considerable diffuse scattering tending to mask the diffraction arcs so that, after preliminary experiments, the strips of cambium were first extracted for 90 minutes with 0.1 N HCl and then built into a block as described above. The only visible effect of this treatment on the X-ray diagram was the clearing up of the background, with no effect on the position of the diffraction arcs themselves.

In determining the micellar orientation, photographs were taken with the X-ray beam directed in turn along three mutually perpendicular axes of the block.

1. Normal to the broad face of the block (*i.e.*, perpendicular to the cell surface and to the longitudinal cell axis, Fig. 1A).

2. Parallel to the broad surface of the block with the beam in the plane of the wall and perpendicular to the longitudinal cell axis, Fig. 1B.

3. Parallel to the broad surface of the block with the beam in the plane of the wall and parallel to the longitudinal cell axis, Fig. 1C.

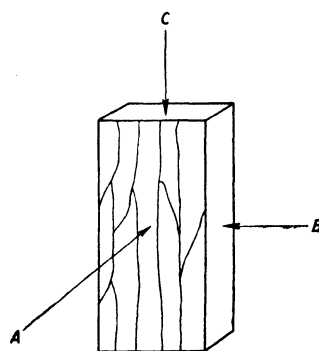


Fig. 1. For explanation, see text

The corresponding X-ray diagrams are reproduced in Plate I, Figs 1A, 1B, and 1C, respectively. In all cases there can be no doubt that cellulose is present in the dried walls and under these conditions is aggregated into micelles though the radial breadth of the arcs suggests that the micelles here are smaller than usual, a point which has been made elsewhere (PRESTON, 1947), and is in agreement with a similar observation by FREY-WYSSLING (1936) in the elongated parenchyma of oat coleoptiles. These considerations apply, it is to be noted, to dried specimens. In view of the work of BERKELEY AND KERR (1946) already mentioned, the condition of wet specimens was briefly examined. When fresh cambium, preserved in dilute aqueous preservatives, was photographed soaking wet then the water haloes were so intense as to mask any cellulose diagram. The presence of water haloes only is no guarantee that the cellulose diagram is absent. When, on the other hand, the material was put into equilibrium with an atmosphere of 98% R.H. and maintained in that condition during X-ray examination, with no further drying at any time, then the intensity of the water haloes was reduced and the normal cellulose diagram was clearly present. The cellulose is therefore organized into micelles under these conditions.

Returning, therefore, to the diagrams of dried specimens the breadth of the arcs makes it difficult to estimate the exact interplanar spacings but Table I gives the average of many determinations made visually and with a photometer. The length of the unit cell along the *b* axis (parallel to the chains) is undoubtedly 10.3 Å and there is no doubt that the spacing of the 002 planes is approximately 3.9 Å. The 10 $\bar{1}$  planes are represented

*References p. 559.*

by an arc corresponding to 5.5 Å, but the diffraction arc from 101 planes is apparently absent. Possible reasons for this will be discussed in a later paper, but it may be noted now that in purified cambial cellulose the arcs corresponding to both these planes are clearly present (Plate I, Fig. 2).

TABLE I  
SPACINGS OF MOLECULAR PLANES IN CAMBIUM

Direction of beam in specimen (see Fig. 1)	Spacings (Å)		
A	3.97	5.54	2.55
B	3.91	5.55	2.55
C	3.97	5.54	2.55

In addition to the arcs already mentioned, others corresponding to spacings of 8.3–8.7 Å were sometimes observed. These can just be detected, for example in Plate I, Figs 1B and 1C. It is difficult at the moment to explain the presence of these anomalous arcs in terms of the accepted structure of cellulose, although preliminary investigations indicate that they are strongly reduced in intensity, or indeed completely absent, in diagrams of cambial cells from which pectins and lipophilic substances have been removed. These arcs are also absent in the diagram of purified cambial cellulose.

As regards the micellar orientation in cambial cells the diffraction photograph corresponding to position 1 above and shown in Plate I, Fig. 1A consists in the main of meridional arcs drawn out into almost complete circles. This diagram, even assuming the cells have not collapsed into flat plates, would indicate the presence of micellar aggregates lying approximately transversely to the length of the cell. This is made clear by consideration of the nature of the spiral diagram. Thus it is well known that the diagram expected from a spiral molecular configuration should consist of arcs in sets of four, symmetrically disposed about the centre of the diagram, and it has recently been shown (PRESTON, 1946) that these fuse into two meridional arcs if the angle of the spiral to the longitudinal cell axis exceeds a certain figure, which depends on the interplanar spacings concerned and the angular dispersion about the direction of micellar orientation existing in the specimen under examination. The figures for undispersed cellulose can be calculated as 73.5°, 81.8° and 82.8° for planes 3.9, 5.4 and 6.1 Å apart respectively. If considerable dispersion exists in the specimen then fusion of the arcs takes place with steeper spiral configurations. Now the outer arcs in Plate I, Fig. 1A correspond to planes of spacing 3.9 Å and the inner arcs roughly to 5.5 Å. The photograph therefore makes it certain that the cellulose micelles in the walls of cambial initials lie more or less transversely to cell length without, however, allowing any precise figure to be estimated. An average value of 80° for the primary walls of conifer tracheids, as found earlier (PRESTON, 1947), would be in harmony with these findings. There can, however, be little doubt that the cells are actually collapsed so that the photograph corresponds to that of a series of crossed crystal plates and the tilt of the micellar direction in either plate (wall) cannot be much less than 80° to the longitudinal cell axis.

The diagrams presented in Plate I, Figs 1B and 1C add some further information.

If the micelles were, in fact, not materially dispersed about their preferred, almost transverse orientation, then Fig. 1B, Plate I, might have been expected to show a series of circles since the X-ray beam would have been parallel to the micelle length. In fact, however, the intensity is much less along the meridian than along the equator. At first sight this could be taken to imply a condition similar to that found in the walls of algae (PRESTON AND ASTBURY, 1937; ASTBURY AND PRESTON, 1940; NICOLAI AND FREY-WYSSLING, 1938; PRESTON AND NICOLAI, 1949), *i.e.*, a preferred orientation of certain molecular planes parallel to the wall surface. In view of the fact, however, that the arc corresponding to planes of spacing 5.5 Å is present in all photographs (Plate I) this condition cannot occur in cambium. The correct interpretation of the photographs in Plate I, Figs 1B and 1C will be clear from the pole figures given, for two possible configurations, in Figs 2(a) and 2(b). Fig. 2(a) shows the condition in which the molecular chains of cellulose are truly transverse to the cell length. The specimen is considered to be located at O in the path of a beam of X-rays  $XOX'$ . A sphere  $P'P''p'p''$ , the projection sphere, is constructed round O as centre and, concentrating on the planes of 3.9 Å

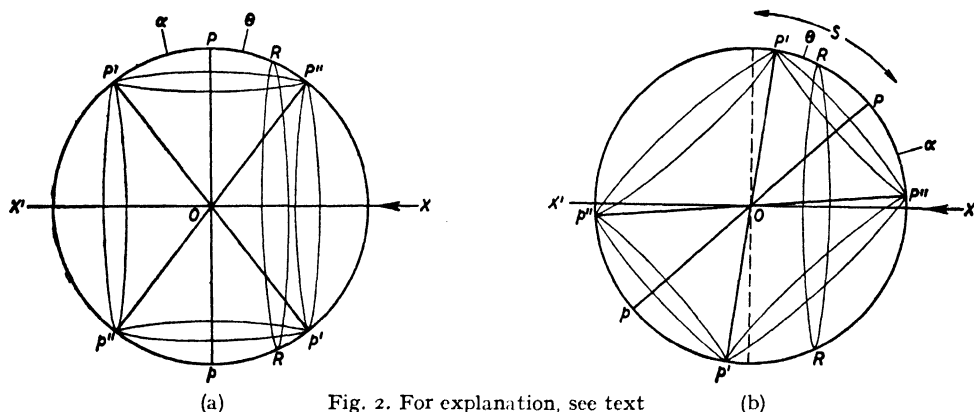
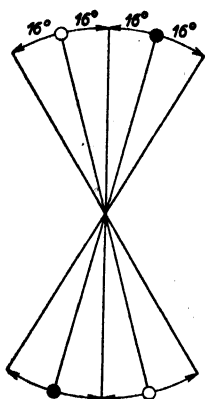


Fig. 2. For explanation, see text

spacing, parallel to the chain length, the normal  $OP$  to these planes in any one transversely oriented micelle is erected from O intersecting the sphere at P,  $OP$  being perpendicular to  $XOX'$ . Since in the real specimen there is, in fact, some angular dispersion about the preferred direction of orientation, as is clear from the presence of an arc of 2.56 Å spacing in Plate I, Fig. 1B, and from other observations described below, the line  $POp$  can be considered to "wobble", defining the solid cone  $P'OP''$  and  $p'Op''$  of half angle  $\alpha$ . If the micelles are completely dispersed around the axis of common orientation this can be taken into account by revolving the cones around the axis  $XOX'$  so that the pole of all the possible positions of the normal becomes a broad band limited by two small circles  $P'p''$  and  $P''p'$ . If the dispersion  $\alpha$  is sufficiently great this band will intersect the reflection circle  $RR$  in a circle, so that the X-ray diagram would be a complete and uniform circle. If, however, the cellulose chains are not truly transverse, but make an angle  $S$  with the transverse direction, then this can be taken into account by tilting the pole figure through an angle  $S$  as shown in Fig. 2(b). It is to be noted that since the configuration is spiral, opposite walls in a cell will be tilted in opposite directions so that two pole figures are required. Only one of these is drawn in Fig. 2(b) since the presence of the other, tilted in an anticlockwise direction through an angle  $S$ , would make the diagram unduly complicated. Under these conditions the intensity in the

region of the point R is reduced. In fact, if  $S > \Theta + \alpha$  the intensity near R would be zero. In the diagram Plate I, Fig. 1B the intensity along the meridian is, in fact, strongly reduced, but not to zero. It may therefore be said that the cellulose chains are tilted to the transverse plane by an angle which is greater than zero but less than  $\Theta + \alpha$  or, approximately for the planes of 3.9 Å spacing,  $11.50 + \alpha$ . The spread of the arcs in Plate I, Fig. 1A shows that the dispersion is not greater than  $\pm 32^\circ$  so that these considerations show that the inclination of the chains to the transverse is less than  $43^\circ$ .



Since, further, a dispersion about the chain direction of front and back walls of  $\pm 16^\circ$  would give a total spread of the arcs of  $\pm 32^\circ$  (Fig. 3) it is apparent from further examination of Plate I, Fig. 1A that the angle of inclination of the micelles to the transverse is not greater than  $16^\circ$ . The three photographs of Plate I, Fig. 1 are therefore, mutually consistent with this figure.

Fig. 3. Diagrammatic interpretation of the X-ray photograph of cambium with the beam normal to wall surface (Plate I, Fig. 1A). The solid circles represent the mean positions of the 3.9 Å arcs corresponding to upper walls of the cells and open circles the corresponding position in lower walls. An angular dispersion of  $\pm 16^\circ$  can then give a meridional arc of total extent  $\pm 32^\circ$ , as observed on the photograph. If the angular distance of the circles from the meridian is greater than the  $16^\circ$  shown here, then the arc would be resolved into two.

TABLE II  
THE BIREFRINGENCE IN TRANSVERSE SECTION OF CAMBIUM AND THE PRIMARY WALLS OF DIFFERENTIATING TRACHEIDS FROM *Pinus sylvestris*

Specimen	Phase difference* (degrees)	Section thickness ( $\mu$ )	Birefringence
<i>Cambium</i>			
Radial Walls . . . . .	8.3	8.0	0.001 <sub>7</sub>
Tangential Walls . . . . .	7.8	8.0	0.001 <sub>6</sub>
<i>Primary Walls of Differentiating Tracheids</i>			
Radial Walls . . . . .	8.3	8.0	0.001 <sub>7</sub>
Tangential Walls . . . . .	8.3	8.0	0.001 <sub>7</sub>

\* Averages of 20 measurements

These conclusions are supported by optical evidence such as that of KERR AND BAILEY (1934) which shows that the cambium is birefringent in both longitudinal and transverse section, an observation which has been repeated during the present investigation and which suggests that considerable angular dispersion of the micelles occurs about their preferred orientation. This is supported also by the magnitude itself of the birefringence of the cambium. From Table II it is clear that the birefringence is of the order of 0.001 to 0.002 in transverse section. If the cellulose content of the wall is taken as 25% (ALLSOPP AND MISRA, 1940) then the observed value is still considerably lower than might be expected. For, assuming the usual mixture formula (see, e.g., HERMANS, 1946) to hold and that the remaining 75% of the wall has a refractive index of  $n_r$  then, ignoring small density corrections,

$$n'_y = 0.25n_y - 0.75n_r$$

$$n'_a = 0.25n_a - 0.75n_r$$

and

$$n'_y - n'_a = 0.25(n_y - n_a)$$

References p. 559.

# PLATE I

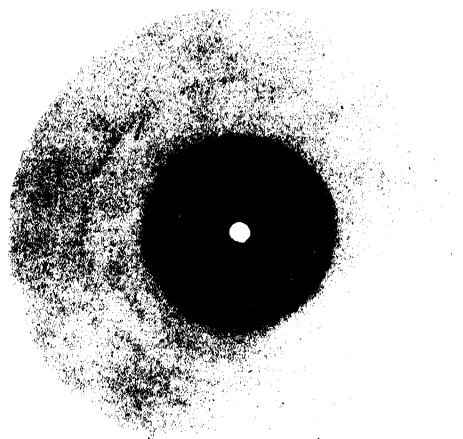


Fig. 1A. Beam normal to cell wall surface, length of initial parallel to length of page (see Text Fig. 1A). Specimen-film distance = 2.86 cm

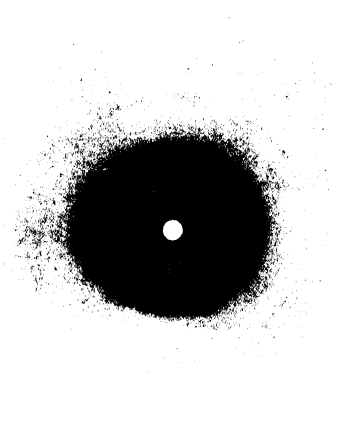


Fig. 1B. Beam parallel to wall surface and perpendicular to longitudinal axis of cells. Cell length parallel to longer edge of page, (see Text Fig. 1B). Specimen-film distance = 2.54 cm

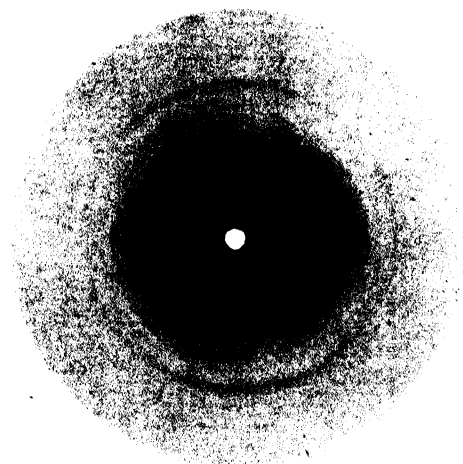


Fig. 1C. Beam parallel to wall surface and to longitudinal cell axis. Wall surface parallel to longer edge of page, (see Text Fig. 1C). Specimen-film distance = 2.86 cm

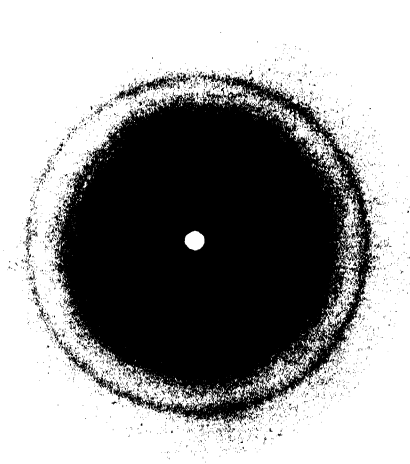


Fig. 2

Fig. 1A, 1B, 1C. X-ray diagrams of dried cambial tissue.  $\text{CuK}\alpha$  radiation, flat film.

Fig. 2. X-ray diagram of a pellet of purified cambial cellulose. Note the presence here of diffraction rings corresponding both to planes of 6.1 Å and 5.4 Å spacing (inner two circles) whereas only the 5.5 Å ring is present in Fig. 1





where  $n_v' - n_a'$  is the birefringence to be expected in a body containing 25% cellulose whose intrinsic birefringence is  $(n_v - n_a)$ . Taking the angle of the chains to the transverse to be  $16^\circ$  which is the greatest possible, then  $(n_v - n_a)$  should be of the order 0.047, assuming the micelles are all parallel and hence

$$n_v' - n_a' = 0.012$$

approximately. This gives the minimum possible figure for undispersed cellulose. Since the real figure is 0.001 to 0.002 there must be either a large angular dispersion or a low crystalline to non-crystalline ratio, or both. The X-ray diagrams show clearly that the former at least is involved.

The above X-ray and optical evidence may be interpreted diagrammatically as in Fig. 4 representing a type of micellar organization essentially similar to that described by FREY-WYSSLING (1930) on the basis of optical evidence and termed by him "Tube Structure". The present investigation provides the only complete X-ray evidence demonstrating such a structure though the writers have applied the same technique in demonstrating this structure in the parenchyma of oat coleoptiles (WARDROP AND PRESTON, 1949).

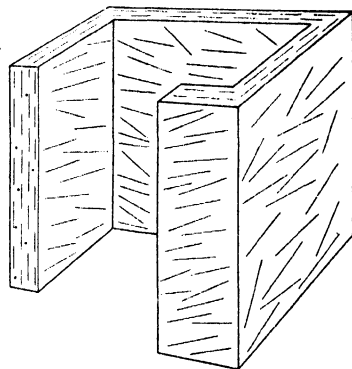


Fig. 4. Diagrammatic representation of a portion of a cambial initial with parts of two walls removed. The lines represent the orientation of the micelles in the walls.

#### THE INTERMICELLAR SYSTEM IN CAMBIUM

The organization of the crystalline portion in cambium cell walls may be taken therefore as fairly well understood. Since, however, some 75% of the weight of the tissue is non-cellulosic it would seem profitable to examine briefly the main features of this non-crystalline material. The chemical analysis of the cambium carried out in this laboratory by ALLSOPP AND MISRA (1940) and that of oat coleoptiles by BONNER (1935) are presented in Table III.

TABLE III  
THE MAJOR CONSTITUENTS OF CAMBIUM AND OF *Avena* COLEOPTILES

Specimen	Cellulose %	Pectin %	Hemicellulose %	Protein %
Conifer Cambium . . . . . (ALLSOPP AND MISRA, 1940)	25.1	16.6	ca. 6	20.8
<i>Avena</i> coleoptile . . . . . (THIMANN AND BONNER, 1933)	42	8	38	12

In cambium the cellulose constitutes only one quarter of the dry weight of the wall substance while in *Avena* coleoptiles it is present to a considerably greater extent. Greater interest, however, centres on the relative volume of the cellulose in fresh material. The fact that, on drying, the wall of each type of cell shrinks to about  $\frac{1}{3}$  of its thickness when wet suggests that this volume is rather low, and FREY-WYSSLING (1936) estimates that in the parenchyma of oat coleoptiles the cellulose occupies approxi-

mately 14% of the wall volume. Similar estimates for wet cambium would suggest a figure of approximately 8%. From this figure it is possible to estimate the order of magnitude of the distance separating the micelles. If, for instance, it is assumed that all the cellulose occurs in micelles of  $d$  Å (circular) diameter separated uniformly by a distance of  $m$  Å, then in a  $1 \text{ cm}^2$  area perpendicular to micelle length there will be  $(1/m)^2 \cdot 10^{16}$  micelles, each  $\pi (d/2)^2 \cdot 10^{-16} \text{ cm}^2$  in cross-sectional area. In a cube  $1 \text{ cm}$  deep, therefore, the total micelle volume will be  $(\pi d^2/4 m^2)$ , assuming the micelles to be at least  $1 \text{ cm}$  long. This is the relative volume of the cellulose and hence

$$(\pi d^2/4 m^2) = 0.08$$

and therefore

$$m = 3.3 d$$

It will be shown in a later paper (WARDROP AND PRESTON, 1949) that  $d$  is of the order of 20–30 Å and so the intermicellar distance would be of the order of 70–100 Å. If, however, it is assumed that only 60% of the cellulose is crystalline then this distance will be increased by  $\sqrt{5/3}$  and would then be of the order 90–120 Å. While this latter condition is most likely to attain, it is probable that the actual distances are very variable.

It is perhaps also interesting to note that if the wall could be considered as consisting of individual cellulose chains arranged strictly parallel to, and equidistant from, each other then the distance between them would be of the order of 13 Å. This is of the order of three molecular diameters and compares favourably with the bimolecular separating films suggested by BERKELEY AND KERR (1946). If the cellulose chains are indeed spun in the surface of the cytoplasm in regular distribution this might be expected as the distance separating them.

#### DISCUSSION

From the foregoing it will be seen that in cambial cell walls the micellar organization approximates to the tube type of FREY-WYSSLING (1930) and is characterized further by the relatively small volume occupied by the skeletal micellar system. It is also of interest that in the absence of lignin there is a close association between the various cell wall constituents although as yet there is no definite evidence of linkages of a chemical kind existing between them. Further the cell wall is intimately related to the cytoplasm as shown by the staining reactions of cambial tissue\*. There is already a good deal of evidence in the literature suggestive of such a relation in the walls of growing cells, notably in the demonstration of the difficulty of plasmolysis of meristematic cells and in the fact that in the growing terminal cells of algae and fungi, although the cytoplasm does move away from the wall in the regions of the filament removed from the apex, in the actual region of growth the cytoplasm adheres firmly to the wall. The physiological significance of the observations most probably lies in the fact that cells which are capable of extension growth usually possess tube structure. In this discussion it is useful therefore to explore what correlations may exist between the structural and physiological aspects of extension growth.

In the first place it would appear unwise to envisage any rigid separation between the wall of the cambial cell and its cytoplasm, at least in the sense used in cells capable

\* These staining reactions will be discussed in a later paper on primary walls generally.

of ready plasmolysis. In this regard, then, it is possible that the wall itself represents the boundary of the cytoplasm which ramifies and interpenetrates the cellulose micelles of which its structural skeleton is composed.

Further support for this view may be found in the organization of the cellulose skeleton itself and of its relation to cell dimensions. The micellar orientation of the wall in growing cells is usually maintained approximately transverse to the longitudinal cell axis irrespective of the cell length. This is suggested by the results of MAAS GEESTERANUS (1941) on the stellate pith cells of *Juncus* and of others, (see FREY-WYSSLING, 1948) and is in sharp contrast to the condition attained in secondary walls. Thus PRESTON (1934, 1948) has shown in the case of conifer tracheids that the micellar orientation is such that as the cells become longer the micellar spiral in the secondary wall becomes steeper and the same has now been found true with some fibres (PRESTON AND MIDDLEBROOK, 1949, PRESTON AND SINGH, 1949). Hence it seems legitimate to infer a relationship between wall and cytoplasm different in primary from that obtaining in secondary walls.

As regards the suggestion of CASTLE (1937) and of VAN ITERSON (1946) and others, that membrane tension in cylindrical cells is the factor governing micellar orientation it is to be pointed out that such orientation could arise in isolated cells, as for example, in algal and fungal filaments and the stellate pith cells of *Juncus*, only from strains operating in the growing cell which accompany dimensional changes during growth. However, in the bulk of the cells investigated the actual dimension changes during growth are in a direction perpendicular to the direction of micellar orientation, that is the cells extend in length rather than in diameter, whereas cambium is one of the few cases where during differentiation the strain operates in the direction of micellar orientation. In the differentiating cambium there is then a case where conceivably strains arising during growth could govern micellar orientation. Even in this case there are serious objections. In the first place, as the above investigations show, orientation exists in the cambium before dimensional changes accompanying differentiation occur. In particular the longitudinal tangential wall of the fusiform initials do not extend laterally, at any stage of differentiation. Nevertheless these walls are largely responsible for the diagrams presented above so that here, too, the micellar orientation is almost transverse. This is a clear demonstration that some factor other than simple strains in the cell wall must govern orientation. Secondly, the cells of the cambium are not isolated but are closely packed, so that the osmotic forces of the cell will not be wholly resisted by the cell wall and will be counteracted by similar stresses operating in adjacent cells.

All the above considerations point to the underlying significance of the cytoplasm as the factor governing orientation and extension of the cell wall. Nevertheless extension of the cells must involve molecular displacement under conditions such that the net micellar orientation is not materially affected. It would therefore be of considerable interest to enquire how far the cell wall structure envisaged in the present discussion will allow displacements of this kind.

In view of the fact that the bulk, though not all, of the work described in this paper has been carried out on dried material, it becomes pertinent to look into the evidence presented by BERKELEY AND KERR (1946) that the chain molecules of cellulose, even in the secondary walls of cotton hairs, while oriented with respect to one another are not aggregated into micelles. It was further suggested that under these conditions the cellulose molecules were separated by bimolecular water films so that no X-ray diffrac-

tion pattern was obtained such as characterizes crystalline substances. In our present investigation the X-ray diffraction pattern of fresh cambium was examined at different relative humidities and in all cases up to 98% R.H. the cellulose was clearly present in micellar aggregates. A preliminary note on this point, including similar observations on algal cells, has already been given (PRESTON, WARDROP, AND NICOLAI, 1948).

The possibility that in growing cells the cellulose skeleton is not organized into micelles is, in fact, not new for it was suggested by HEYN (1940) some years ago. Unfortunately it is difficult to assess the merits of the idea as proposed by BERKELEY AND KERR (1946) in terms of the X-ray diagrams they present. Although the same fibre bundle was used in the observations presented in their paper (Fig. 2, p. 305) it is not explicitly stated that the specimen was exposed to X-rays for the same period under the different conditions. This leaves open the possibility that in the fresh material the water halo was of sufficient intensity to mask completely the X-ray diffraction pattern of cellulose, and in this regard it is to be noted that quite generally fresh cellulose material has a higher water content than it has after drying and rewetting. This effect may well be exaggerated in the present case since in the fresh material, at least up to the time of cessation of secondary thickening the lumina of the cells are water filled whereas after drying and rewetting it is possible that the (collapsed) lumina would not refill with water. This is, in fact clear from the diagram in Fig. 2 of their paper since the dried, rewetted bundle shows no water halo at all. The complete absence of a water halo here is indeed puzzling. There is again the further point that in fresh material the micelles may be, and probably are, more dispersed around their common direction of orientation than after drying. The X-ray diagram would then be more diffuse and more easily masked by the water halo. This may also explain the appearance of a cellulose diagram in wet cotton after stretching, for elongation would again reduce the dispersion. Thus it seems that any conclusions based upon these diagrams are at least open to question. In any case the complete absence of diffraction arcs does not necessarily imply complete separation of the molecular chains of cellulose, for according to FANKUCHEN AND MARK (1946) diffraction arcs are not to be expected from micelles less than a certain diameter (*ca* 20 Å).

If, as our evidence suggests, the cellulose is always aggregated into micelles in the cambium then the micellar structure must be sufficiently flexible to allow considerable dimensional changes to occur. This point will, however, be discussed in a later paper.

#### SUMMARY

An X-ray and optical examination has been made of the walls of cambial initials in conifers, and it has been concluded that the structure of these growing cells approximates to the tube structure of FREY-WYSSLING. This is the first demonstration of this structure made by X-ray methods. The molecular chains of cellulose are inclined to the transverse at an angle which is less than 16° but greater than 0°, and the "micelles" are narrower than in secondary walls and have considerable angular dispersion about their common direction of preferred orientation. X-ray examination of fresh cambium at high relative humidities indicate that the same structure obtains in the fresh tissue.

#### RÉSUMÉ

L'examen optique et l'étude par les rayons X des parois des cellules terminales du cambium chez les conifères a montré que la structure de ces cellules en voie de croissance se rapproche de la structure des tubes de FREY-WYSSLING. C'est la première démonstration de cette structure faite par les rayons X. La chaîne moléculaire de cellulose forme avec la perpendiculaire à l'axe de la

cellule un angle compris entre  $0^\circ$  et  $16^\circ$ ; les "micelles" sont plus étroites que dans la formation secondaire et présentent une dispersion angulaire considérable autour de l'orientation générale. L'étude par les rayons X du cambium frais à forte teneur en eau montre que la même structure existe dans les tissus frais.

### ZUSAMMENFASSUNG

Die Wände der Endzellen des Kambiums der Koniferen wurden optisch und mit Röntgenstrahlen untersucht und es wurde gefunden, dass die Struktur dieser wachsenden Zellen der Röhrenstruktur von FREY-WYSSLING ähnelt. Dies ist der erste Nachweis einer solchen Struktur mit Röntgenstrahlen. Die Zellulosemolekülkette bildet mit der Senkrechten zur Achse der Zelle einen Winkel von  $0^\circ$  bis  $16^\circ$  Spannweite; die "Mizellen" sind schmaler als in den sekundären Wänden und zeigen starke Abweichungen rund um die allgemein bevorzugte Richtung. Eine Röntgenstrahlenuntersuchung des frischen Kambiums mit starkem Feuchtigkeitsgehalt zeigt, dass frische Gewebe die gleiche Struktur besitzen.

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## BOOK REVIEW

*Suggestions for science teachers in devastated countries.* Prepared by J. P. STEPHENSON, 88 pages; published in 1948 by the United Nations Educational Scientific and Cultural Organization, Unesco House, 19, Avenue Kleber, Paris (16).

This little book will be welcomed by every science teacher in devastated countries. Written by a science master at the City of London School who is a member of the Royal Society Committee for Co-operation with Unesco, the booklet explains how science teaching can be started with equipment improvised from materials which are easily obtainable. Information is provided for the construction of simple apparatus in astronomy, weather studies, physics, chemistry, and biology; for the arrangement of class and demonstration lessons including discussion and pupils' experiments, and class exhibition work.

There are chapters concerned with visual aids in science teaching, recent laboratory materials, and laboratory receipts, charts and logarithm tables. The aim of the book is practical. It gives not only concrete suggestions illustrated by excellent diagrams, but also stimulates the reader to make further improvisations in reaching practical science. The Ministries of Education of Poland, Czechoslovakia, Greece, Austria, Hungary, Italy, China and the Philippines are free to reproduce this book in English or in translation.

J. W. MEDUSKI, Warsaw (Poland)

## THE ELECTRON MICROSCOPY OF F-ACTIN

by

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Through its ability to portray particles of macromolecular dimensions, the electron microscope is affording a new approach to problems of the fine structure of muscle. Of the two possible methods of attack, one deals with the fine structure of intact muscle fibres, the other with the macromolecular particles of proteins that can be extracted from muscle. We have been employing both methods in combination with one another and with developing chemical studies. This paper records observations on the fine structure of actin as one of the proteins isolated from muscle.

Evidence for the existence of actin was first obtained by BANGA AND SZENT-GYÖRGYI<sup>1</sup>, who found that the solution prepared by extracting muscle for 24 hours with Edsall's salt solution had an extraordinarily high viscosity. This actin, as isolated by methods worked out by STRAUB<sup>2</sup>, has exhibited properties that have made it the classical example of a substance showing a globular-to-fibrous (G-F) molecular transformation. Isolated from muscle dried with acetone, it is a protein of low viscosity which when made 0.1 M with respect to KCl and allowed to stand becomes more viscous and ultimately turns into a thick thixotropic gel. Everything that has since been learned about this change has confirmed the interpretation of SZENT-GYÖRGYI AND STRAUB that actin does indeed occur in two forms, a globular and a fibrous modification.

Two electron microscopic investigations, by JAKUS AND HALL<sup>3</sup> and by ASTBURY, PERRY, REED, AND SPARK<sup>4</sup>, have revealed the particles of F-actin as long, structureless threads; beyond indicating that their molecules must be small, all results on G-actin have been equivocal. In this article we are concerned with a possible fine structure in the F-actin threads, with the details of how they arise from globular actin and with the relation between them and particles of similar dimensions that can be seen in muscle fibrils.

In all the present experiments globular actin was extracted from excised rabbit muscle according to GUBA's modification of the method of STRAUB. Actin threads were made by adding 0.1 M KCl to a solution of this actin and waiting for the G-F transformation to take place at room temperature. In a first set of experiments drops of this transformed actin were put on collodion covered grids, the excess was withdrawn and salt was removed by washing with distilled water; these cleaned preparations were then shadowed with chromium or palladium and examined in an RCA-type EMU electron microscope. A concentrated actin solution gave the kind of network described by ASTBURY, PERRY, REED, AND SPARK. Where fine threads could be observed among its

\* Special Fellows, National Institutes of Health, U.S. Public Health Service.

*References p. 569.*



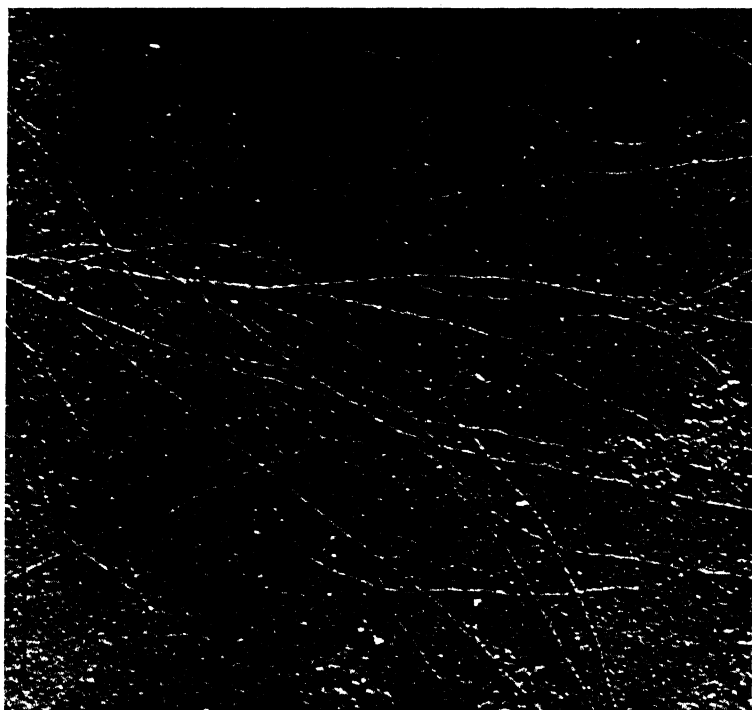


Fig. 1. Separate fibrils of F-actin resting on a collodion substrate. Magnification = 29000  $\times$

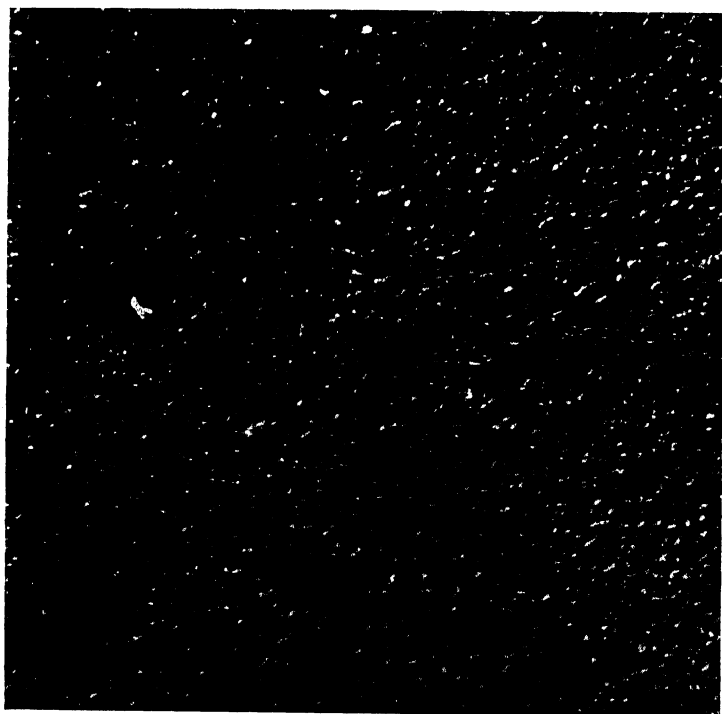


Fig. 2. Sheaves of F-actin beginning to form in a thick mass of G-actin.  
Magnification = 25000  $\times$

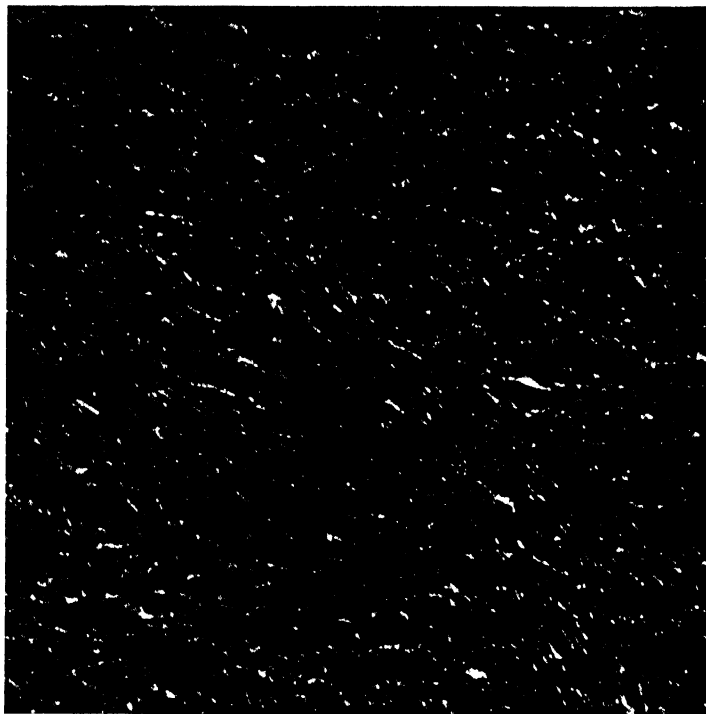


Fig. 3. A preparation similar to that of Fig. 2, seen at higher magnification ( $43\,000\times$ )

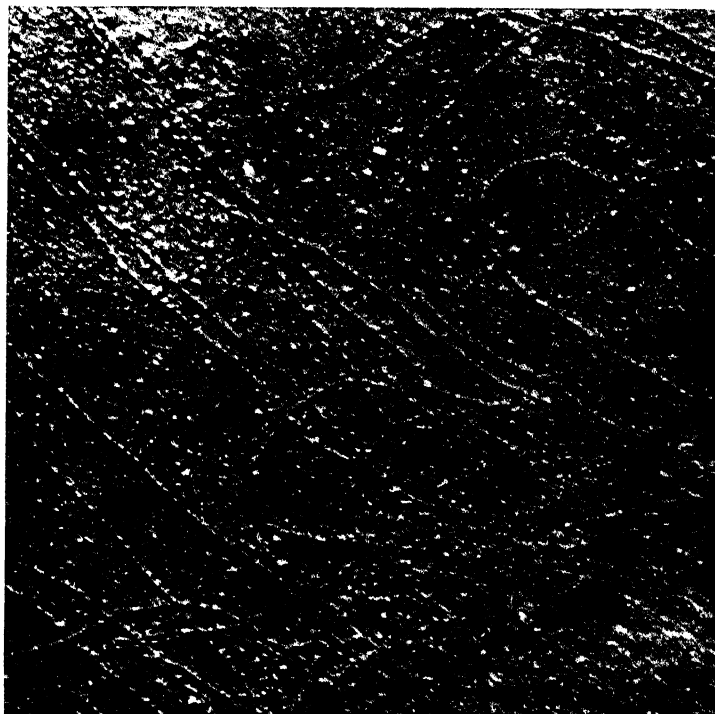


Fig. 4. Single fibres of F-actin extending from a mass of F-actin resulting from the *in situ* conversion of G-actin. Magnification =  $45\,000\times$

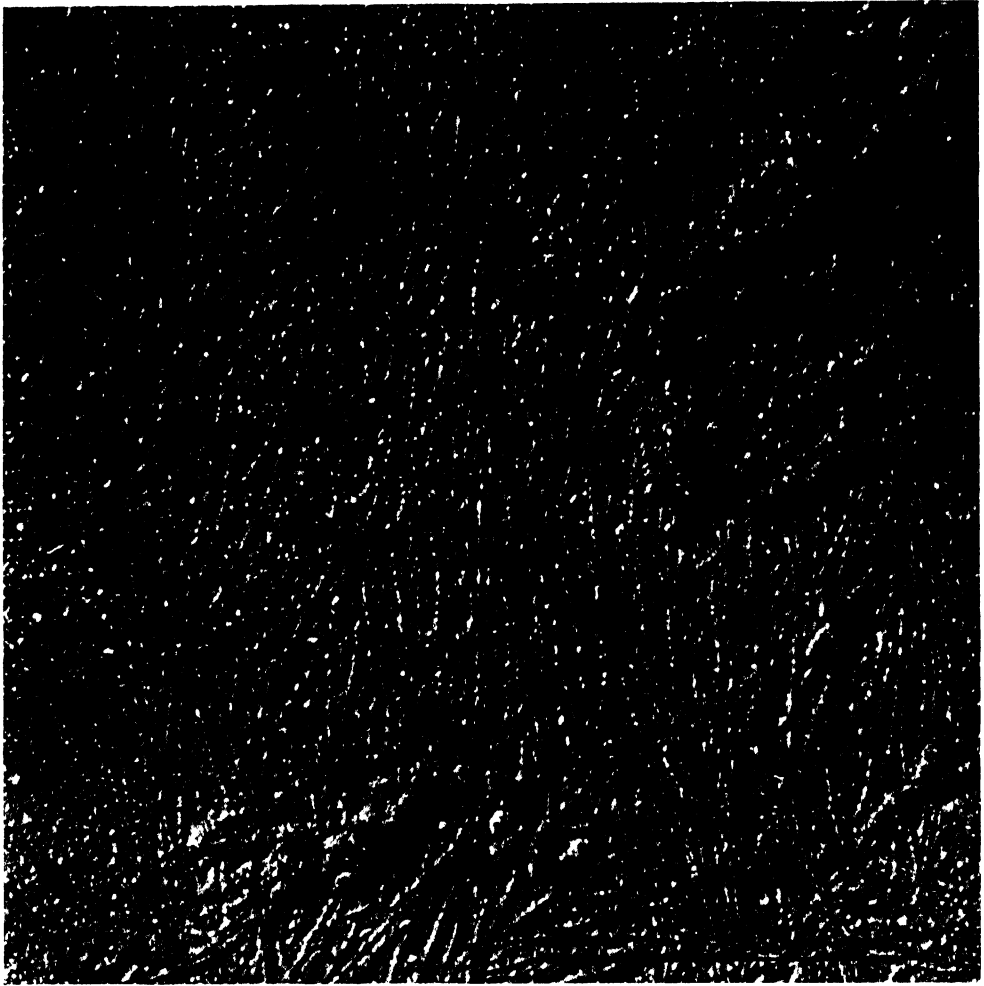


Fig. 5. Groups of F-actin fibres formed *in situ* by conversion from G-actin. Magnification --- 38000  $\times$

bundles, no detail was seen within them. A more dilute actin solution resulted in preparations (Fig. 1) containing very long fine threads with the dimensions of those pictured by JAKUS AND HALL. These individual threads were so thin that roughness of the collodion substrate interfered with their portrayal and confused any attempts to observe their fine structure.

In the effort to avoid this difficulty another set of experiments was made in which the transformed actin was deposited on a microscope slide, washed, shadowed with palladium and removed as a "pseudo-replica" with the help of the usual reinforcing collodion film. Unfortunately, these "pseudo-replicas" were not more successful than direct deposits on collodion in revealing fine structure within actin threads.

In a third and more rewarding group of experiments, the G-F transformation was carried out directly on the glass slide which could then be "replicated" without any disturbance of the fibrous structure as formed. To do this, freshly extracted globular

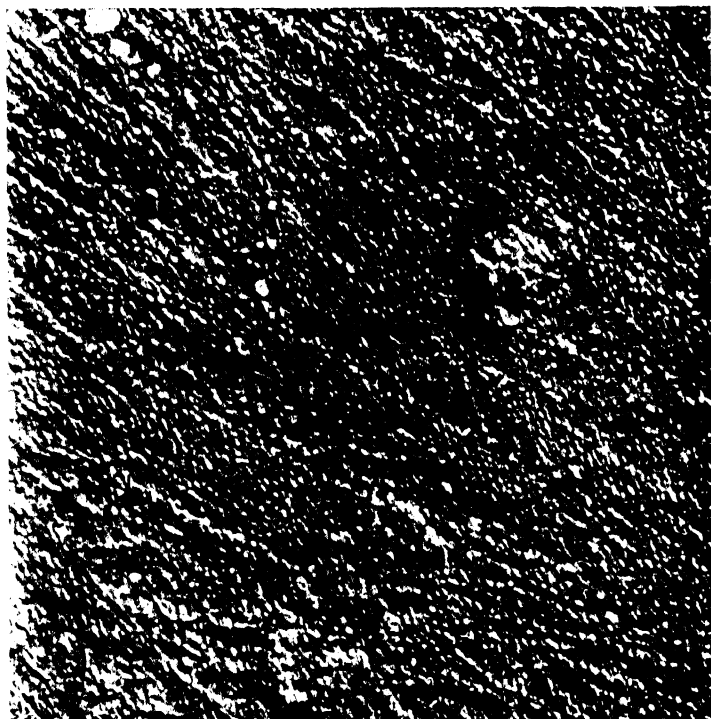


Fig. 6. Another field in an F-actin preparation formed *in situ* from G-actin. Magnification = 50000  $\times$

actin containing a minimal amount of KCl instead of the previous 0.1 M quantity was dropped onto the slide and allowed to evaporate. At a certain point in this evaporation, the small amount of salt present reached the necessary concentration and fibrous actin formed on the slide. Interfering KCl crystals have rarely been found in such preparations and the threads have not been disturbed after their formation by the pipetting and stirring involved in handling preformed fibres.

Various stages of fibre formation can be observed in preparations made in this way. An initial step in the transformation to fibres is illustrated in Fig. 2 where threads can be seen developing in sheaves from a few points of origin. The single threads in these bundles appear against a background whose unevenness is due to its being a thick mass of untransformed globular actin. Fig. 3 shows at a higher magnification a slightly more advanced state in the transformation. Detail cannot be seen within the fibres of either photograph, presumably because the preparations are very thick.

There is less interference from globular actin as the transformation proceeds; if less concentrated globular actin solutions are used, preparations ultimately will be obtained that show only the fibrous form (Fig. 4). The fibres thus prepared have a uniform thickness of about 100 Å; they are very long, do not branch and do not split into finer threads at their ends. Most isolated threads seem devoid of internal structure, but in places at the lower right of this photograph there are indications of their being built of units *ca* 300 Å long whose long axes do not necessarily coincide with the fibre axes.

A conspicuous cross striation appears when the transformation takes place under

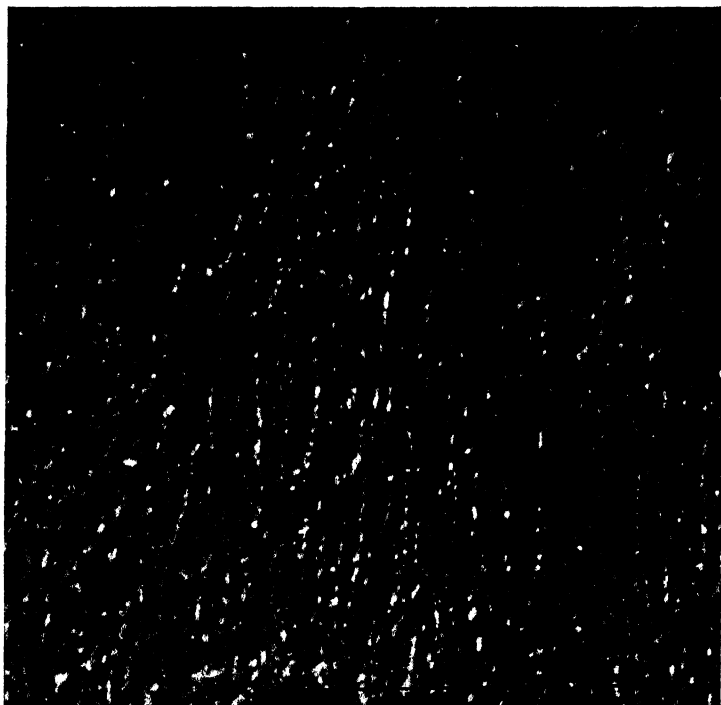


Fig. 7. A part of the preparation of Fig. 5 seen at a higher magnification ( $55\,000\times$ )



Fig. 8. Another preparation of F-actin formed *in situ*. Magnification =  $41\,000\times$

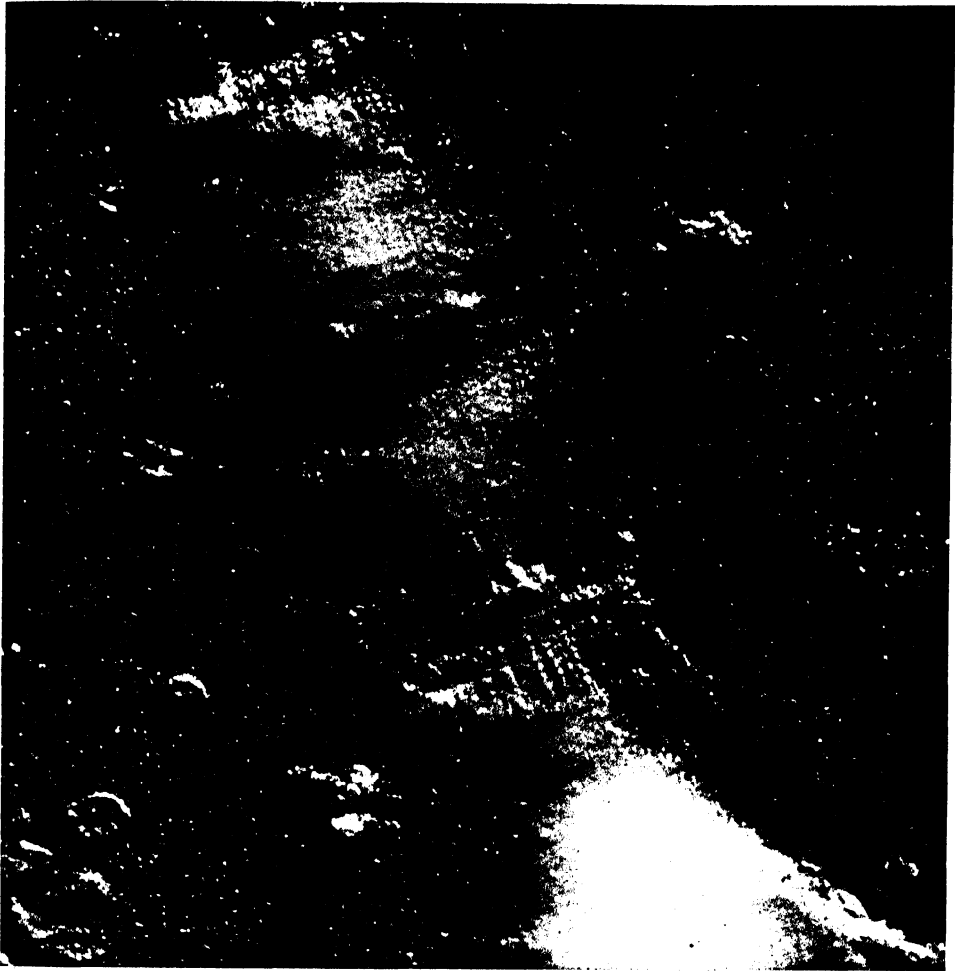


Fig. 9. Part of a minute, intact fibril of rabbit muscle showing its macromolecular fibrillar structure.  
Magnification  $\times 37000$

conditions such that the resulting fibres are in contact to form parallel closely packed aggregates. The cross striation is seen when no more than two threads are in contact but is more evident in broader bundles whose cross segmentation and lateral association are often so orderly as to give the impression of a regular net (Fig. 5). Frequently the cross bands are more conspicuous than the original fibres (Fig. 6).

The single actin filaments seemingly are lengthwise associations of ellipsoidal rodlets *ca* 300 Å long and *ca* 100 Å wide. The cross striation is the result of a regular side-by-side association of these particles (Figs 5 and 7). In many photographs this cross banding stands at an angle of  $90^\circ$  to the original fibre axis but in most pictures its angle is smaller, down to  $20^\circ$ . In many fields all fibres show about the same angle. Fig. 6 is a further good example of this two-dimensional regularity in arrangement.

Many fibres, such as those shown in Figs 7 and 8, give the impression that the common axis of the parallel particles composing them may be inclined to the fibre axis.

*References p. 569.*

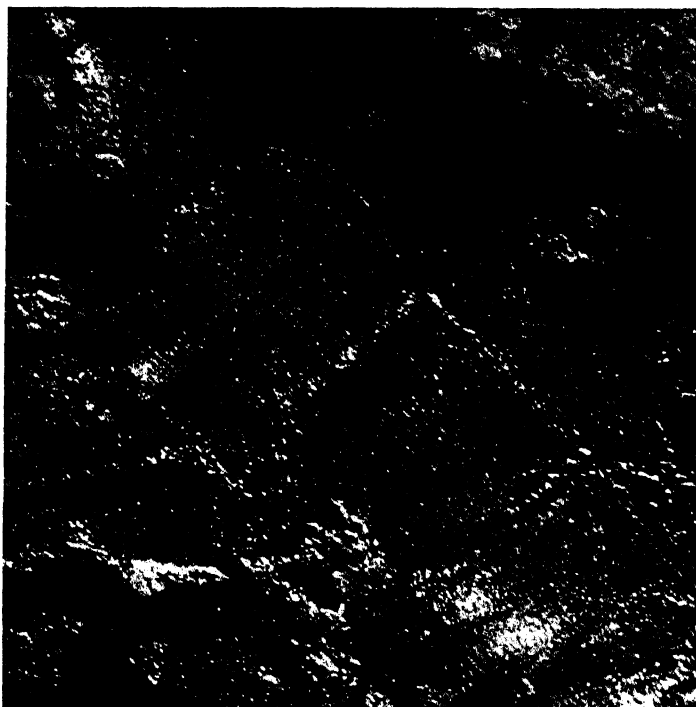


Fig. 10. A portion of another intact muscle fibril from the rabbit. Magnification = 29 000  $\times$

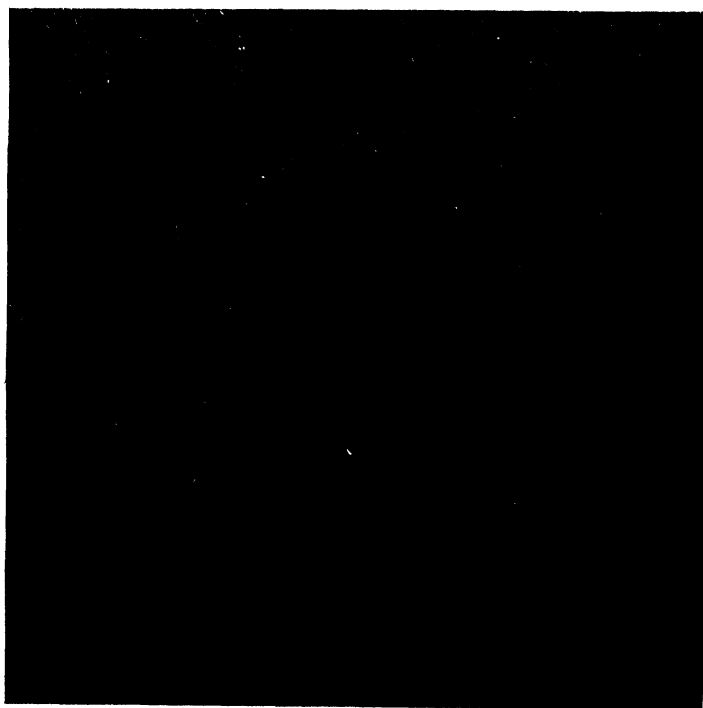


Fig. 11. Elementary fibrils obtained by the mechanical disintegration of muscle fibres such as those of the two preceding figures. Magnification = 30 000  $\times$

The lengthwise association of the particles of a fibre would then be not strictly end-to-end but intermediate between end-to-end and side-by-side.

It is important to compare these results with what is known of the size of the actin molecule. An ellipsoidal protein particle having the dimensions of the segment seen in these electron micrographs (*ca* 300 Å by *ca* 100 Å) would have a molecular weight of *ca* a million and a half. This is much bigger than the known weight of the molecule of globular actin which according to both STRAUB<sup>6</sup> AND SNELLMAN, TENOW AND ERDÖS<sup>7</sup> is *ca* 70000. Results are understandable, however, if the real G-F transformation in actin is a polymerization of 70000 weight molecules to form the ellipsoidal elements seen in the electron micrographs and if these polymerized units then invariably associate together to form fibres and bundles.

The observations of fine detail in F-actin deposits raise questions as to their bearing on the structure of muscle. Intact fibrils small enough to reveal their macromolecular constitution appear as in Figs 9 and 10. Evidently they are in large measure bundles of filaments that have the overall dimensions of the F-actin threads. If bits of muscle are disintegrated in a Waring blender these filaments are liberated. After washing they appear as in Fig. 11. The similarity between them and freshly formed threads of F-actin is made clear by comparing this figure with Fig. 4. The threads in the intact muscle of Figs 9 and 10 are for the most part independent of one another, but small regions can be found which exhibit an approach to the two-dimensional order seen in the actin photographs of this paper. We are examining further this implication that the filaments in intact muscle may be related to the corresponding particles of F-actin.

#### SUMMARY

Electron micrographs have been made of deposits of F-actin polymerized *in situ* from the globulin form. Aggregates of fibers obtained in this way show a new cross segmentation and lateral association which indicates a fine structure within the single fibers.

#### RÉSUMÉ

On a fait au microscope électronique des photographies de sédiments de F-actine polymérisée *in situ* à partir de la forme globuline. Les agrégats de fibres obtenus de cette manière montrent une nouvelle segmentation croisée et une association latérale, qui indiquent une structure fine à l'intérieur des fibres simples.

#### ZUSAMMENFASSUNG

Elektronen-Mikrogramme wurden aufgenommen von *in situ* aus der Globulinform polymerisierten Ablagerungen von F-Aktin. Fiber-Aggregate, die auf diese Weise erhalten werden, lassen eine neuartige gekreuzte Unterteilung und eine seitliche Vereinigung sehen, welche eine Feinstruktur innerhalb der einzelnen Fibern zeigen.

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## OBSERVATIONS MADE WITH THE ULTRAVIOLET MICROSCOPE ON THE MINOR SPIRAL OF CHROMOSOMES IN *OSMUNDA*

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The ultraviolet microscope, in the present context, has been used as a high resolution instrument comparable to an extra lens on the visual light microscope and not, as is often the case, as a form of microspectrometer for the chemical identification of cell components. From numerous studies by other investigators in the latter field the powerful absorption of ultraviolet light by nucleoproteins is well known. This means that the fine details of chromosome structure are a particularly suitable field for high power microscopy with ultraviolet light since the intensity of photographic contrast obtainable by this means is comparable to that conferred by the use of stains in visual microscopy, while the greater degree of optical resolution corresponding to the reduction of wave length from visual light to ultraviolet permits of a virtual doubling of significant magnification. This improvement is of course trifling compared with the spectacular powers of the electron microscope but the practical difficulties in the handling of the specimen are also correspondingly less. Except for the replacement of the human eye by the camera and for the greater degree of mechanical precision required in the optical equipment, ultraviolet microscopy is so like visual microscopy that the same type of material in almost the usual condition can be utilized. This means that a preparation which has been closely studied with visual light and in which significant detail near the limit of optical resolution is known to exist can be transferred to the ultraviolet microscope and be re-examined with immediate clarification of view once a very few preliminary difficulties have been overcome. Discussion of these difficulties and of the treatment needed to circumvent them for cytological material such as that to be discussed below will be found in MANTON AND SMILES (1943).

The observations with which this paper is concerned were carried out in the summers of 1944 and 1945 after which, for reasons unconnected with the work, it had temporarily to be discontinued. Had this not occurred the particular topic of the minor spiral would have been developed more completely before publication was attempted and some of the photographs which have yielded significant evidence would perhaps have been replaced by others more perfect technically or better suited to mechanical reproduction. Even without this, however, they represent a body of new knowledge which could not have been obtained by the methods prevailing in 1939 and since the field of enquiry is itself only a part of a larger investigation which is about to be resumed under somewhat changed circumstances, incompleteness in the sense of the original programme is perhaps unimportant.

The fern *Osmunda* which has formed the subject of three previous communications

(MANTON, 1939; MANTON AND SMILES, 1943; MANTON, 1945) is the most favourable cytological material which has so far been encountered among the lower plants and though the chromosomes are both smaller in size and more numerous than those of the most frequently studied Dicotyledons and Monocotyledons, its systematic position as well as its intrinsic merits give it a particular interest. That its intrinsic merits are not negligible is shown by the fact that on certain matters, notably the estimations of chromonema length at leptotene and of direction of coiling in the sister chromatids of a split somatic chromosome, the information available for *Osmunda* is more complete than that for any other plant. For this reason alone the filling in of gaps in our knowledge wherever possible is particularly desirable and the ultimate objective of the whole investigation is to integrate the facts for spiral structure, both quantitative and qualitative, into the morphological descriptions of mitosis and meiosis more fully than has previously been done.

#### MATERIAL AND METHODS

Though the methods to be used here are essentially those of MANTON AND SMILES (1943), some differences of detail are involved in the cytological treatment of a different tissue. In 1943 the test object was the germinating spore. In the present paper it is spore mother cells at the second meiotic division. The standard treatment for revealing spiral structure in the chromosomes of either meiotic division is to expose the living cells to ammonia, either as vapour or in solution, for a few seconds before fixation. In the first paper of this series (MANTON, 1939) the ammonia treatment had been applied according to the method of SAX AND HUMPHREY (1934) the end product of which is a balsam mount stained in gentian violet. This method has now been replaced by the following.

A fresh smear of mother cells is covered with a drop of 20% alcohol containing ammonia, for ten seconds, the exact strength of ammonia required being determined by trial. The ammoniated alcohol is then drained off and the cells are killed with a drop of acetocarmine which is almost at once replaced by a second drop to remove admixture with the alcohol. This reagent is then left on for long enough for the chromosomes to become visible and the stage and efficacy of the treatment assessed. They are then transferred to acetic alcohol to harden. Two strengths (1:1 and 1:3) of absolute alcohol: glacial acetic acid mixture are conveniently used for this purpose in the latter of which the preparation may remain for anything up to 12 hours. A new drop of acetocarmine is then put on, a coverslip is added and the preparation heated gently over a flame without being allowed to boil. Gentle manual pressure is then applied by passing a finger over a piece of blotting paper placed over the coverslip. This pressure spreads the contents of cells more nearly into one plane, a feature which greatly assists observation with visual light and is indispensable with ultraviolet. At the same time the action of the reagent tends to enlarge all dimensions of the chromosomes without thereby introducing any other detectable artifact. This also facilitates observation although some of this enlargement is reversible and is lost when the acetic acid is replaced by other reagents. Once the heat and pressure have been applied the preparation is virtually finished and it may either be examined at once with visual light, or it may be treated as an ordinary acetocarmine smear and transferred by stages to balsam, or it may be incorporated in a cellulose nitrate film and transferred bodily to a quartz slide ready for the ultraviolet microscope. The details of the latter process are given in,

MANTON AND SMILES (1943) under the heading of WELCH's Durofix transfer method.

Before ultraviolet photography can begin the cellulose nitrate film must be dissolved away and the stain as far as possible removed by soaking in 45% acetic acid. A quartz coverslip is then put on and ringed with wax, the mounting medium being either 45% acetic acid or any other convenient transparent liquid. Serial photography right through the specimen at known differences of focal level, usually either 0.1 or 0.2  $\mu$  between consecutive exposures, then provides the entire evidence on which observations are based. For the material under discussion a polar view of a plate of chromosomes may contain anything from ten to thirty significant focal levels. For the first working out of the evidence all the focal levels of a successful series are printed by photographic enlargement to a standard magnification of three thousand diameters but for purposes of reproduction in half-tone selected portions of the evidence have been further magnified to four thousand diameters, a degree of enlargement which represents the limit of significant magnification with ultraviolet light in the sense that two thousand or perhaps three thousand is the limit with visual light.

The ultraviolet microscope used has been the Beck-Barnard instrument at the *National Institute for Medical Research, Hampstead*, which carries Zeiss quartz lenses computed to work with the 2750 Å Cadmium line supplied by a stationary electrode cadmium spark source. I am deeply indebted to the MEDICAL RESEARCH COUNCIL and to the authorities of the Institute for their generosity in receiving me as a guest whilst working there. I am also personally indebted to Mr. SMILES and Mr. WELCH for technical help in operating the instrument and to my own technical assistant Mr. CLARKE for preparing the prints for publication. I must also acknowledge the assistance from the ROYAL SOCIETY of a small grant towards the cost of frequent journeys to London, which I held during 1944 and 1945.

#### THE MINOR SPIRAL

The minor spiral in the present context means the spiral revealed by ammonia treatment in the chromosomes of the second meiotic division. In *Osmunda* as in many other plants this spiral differs from that at the first meiotic division (the major spiral) by being narrower in diameter and having a larger number of gyres. It also differs from the spiral of a somatic chromosome in having a smaller number of gyres and therefore it is perhaps important to emphasize the limitation which will be put on the word "minor". It has sometimes by other writers been loosely applied to any manifestation of spiral structure other than the major coil of the first meiotic division; it will, however, on this occasion be strictly confined to the spiral of the second meiotic division only.

A difference in the number of gyres and in diameter, between the spirals at the two meiotic divisions is a very usual though not a universal feature in plants (exceptions include *Trillium* and *Vicia*). Some idea of the extent of the difference may be obtained by a glance at Figs 1-4, which show major and minor spirals in two well known organisms *Tradescantia virginiana* and *Osmunda regalis*, in each case photographed with visual light from a preparation stained in gentian violet after the standard pretreatment of SAX AND HUMPHREY (1934). The photographs are all at the same magnification ( $\times 3000$ ) for the sake of comparability. This degree of enlargement is clearly excessive for the very large chromosomes of *Tradescantia* (Figs 1 and 2) but is necessary for effective reproduction of the essential details of the smallest object, the minor spiral of



Fig. 1. *Tradescantia virginiana* the major spiral photographed with visual light from a balsam mount stained in gentian violet,  $\times 3000$ . Two focal levels of an unpaired chromosome with an optical section of a ring pair above.



Fig. 2. The same showing the minor spiral at anaphase of the second meiotic division, for comparison of size with *Osmunda*.

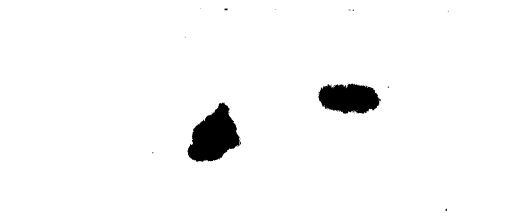


Fig. 3. *Osmunda regalis* the major spiral, treatment and magnification as in Fig. 1.

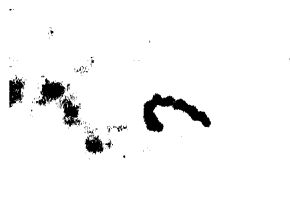


Fig. 4. The same showing the minor spiral (after MANTON, 1939).

*Osmunda* in Fig. 4. These four figures express not merely the relative difference between major and minor spirals in the same plant but also the permanent differences of size between the two species. In both, the major spiral falls easily within the province of visual microscopy. In *Osmunda*, however, the minor spiral is so near to the limit of resolution that the need for special methods for observing it is at once apparent. That even with the new methods the minor spiral of *Osmunda* remains uncomfortably small cannot be denied but comparison of Figs 7 and 9 (et seq.) both with each other and with Figs 2 and 4 respectively will show that the technical improvement though not unlimited is sufficiently definite to make all the difference to the study of this particular problem.

#### CONVENTIONS REGARDING DIRECTION OF COILING

Direction of coiling does not figure largely in the new evidence although some understanding of it is essential, for the reason that unless direction can be demonstrated, in parts at least of the chromosomes under observation, it cannot be certainly known that the features observed are really parts of a spiral. For this reason a word may perhaps be said regarding the conventions used for expressing direction.

As on the previous occasion (MANTON AND SMILES, 1943) the meanings attached to the words right-handed spiral and left-handed spiral will be those of the physical sciences and not those of the biological sciences which use the words in an exactly inverse sense. In the physical sciences a right-handed spiral is that of the ordinary



Fig. 5. *O. regalis*, early second division not treated for spiral structure, to show gross morphology of the chromosomes. Permanent acetocarmine photographed visual light  $\times 1000$  (after MANTON, 1939). Two lost chromosomes in the cytoplasm still show the major spiral of the previous division; other chromosomes long and thin, with minor spiral invisible.

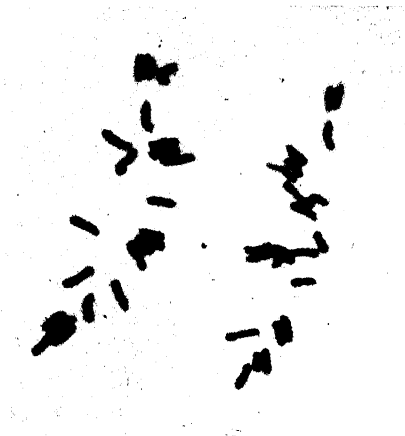


Fig. 6. The same at anaphase of the second division. Note changed shape of the chromosomes though the minor spiral is invisible (after MANTON, 1939).

carpenter's screw and an example of it is visible in Fig. 1 in which (a) is the upper focus and (b) the lower. Had (b) been the upper surface and (a) the lower the spiral would have been left-handed.

Other examples of *right*-handed spirals are the two marked chromatids of the right hand central chromosome of Figs 7, a-d in *Tradescantia*, while a *left*-handed spiral in the smaller chromosomes of *Osmunda* may be seen in the marked chromosome at the bottom of Figs 9a and b. A very clear example of a *change* of direction may be seen in the lower chromatid of the left-hand central chromosome of Figs 7a and b and other examples of all these phenomena will be discussed below.

#### MORPHOLOGY OF CHROMOSOMES AT THE SECOND DIVISION IN *Osmunda*

A glance at Figs 5, 6 and 8 will sufficiently explain the morphology of the chromosomes of *Osmunda* in features other than spiral structure. At the end of anaphase (Fig. 6) the haploid complement of 22 rod shaped chromosomes of very uniform size and shape is easily seen, the position of the centromere in all but one of the chromosomes being approximately terminal. The position of the centromere is better seen at metaphase since it is then revealed as the place at which the otherwise widely diverging chromatids are attached. In *Tradescantia* the median centromeres give the second division chromosomes at metaphase an X shape (Fig. 7). In *Osmunda* with terminal centromeres the shape approximates to that of a V as may be seen in parts of Fig. 5 and again in Fig. 8 and subsequently. The only chromosome in *Osmunda* possessing a more nearly median centromere is that numbered 2 in Fig. 8. This is also the chromosome previously used for demonstration of the minor spiral in Fig. 4 from which it is clear that the short arm is about half the length of the long one. This chromosome is of importance because it is the only one in the haploid complement which can be separately



Fig. 7. *Tradescantia virginiana*. High power U.V. photographs of the minor spiral  $\times 4000$ . Four focal levels  $0.4 \mu$  apart showing details of direction of coiling in parts of two chromosomes. In the right-hand central chromosome both chromatids are coiled in right-handed spiral from the centromere to the right-hand end. The left-hand central chromosome shows a change of direction in the lower chromatid. Fig. a the top focus, Fig. d the bottom. (Series 105 exposures 3a, 4a, 4b, 5b).



Fig. 8. *Osmunda regalis*. General view of the cell used for the early stage of the minor spiral. Low power U.V. photograph  $\times 500$ .

identified, the shapes of all the others being so much alike as to make them for practical purposes indistinguishable.

If half-chromosomes (chromatids) only are considered close comparison between Figs 5 and 6 will make clear another detail of difference, this time between early and late stages of the second division. At early metaphase (Fig. 5) the chromatids are relatively longer and thinner than they appear to be at anaphase (Fig. 6) and this difference can be shown to be caused by changes occurring during metaphase itself, early stages such as Fig. 8 being like Fig. 5, but late stages of metaphase such as those of Figs 16–18 being indistinguishable from anaphase except for the position of the chromatids on the spindle. Demonstration that the basis of this apparent change of dimensions lies in a change in the number and size of the gyres of spiral structure is the main purpose of this paper. It is, however, important to notice that the difference can be demonstrated by all the more usual cytological techniques which do not directly reveal the spiral and there can therefore be no question that the appearance is an artifact.

#### THE MINOR SPIRAL AT EARLY METAPHASE

A general view of the cell from which the best evidence on the early state of the spiral has been derived has already been introduced as Fig. 8 and some enlarged details of individual chromosomes will now be discussed.

Chromosome 1 (Figs 9 and 10) provides a useful introduction to the spiral and two focal levels of it are reproduced in two types of print in the hope that the necessary details will survive mechanical reproduction in one or other if not in both. Fig. 9, on the left of the page, shows two views in ordinary photographic enlargements at a magnification of four thousand diameters, the upper focal level, Fig. 9a being uppermost on the page. Fig. 10, on the right of the page, shows the same two focal levels in negative prints, the image of these being reversed since the negative print is obtained by contact from the positive. In all four prints the upper chromatid is uninterpretable but the lower is far better. It contains about 14 gyres which are fairly evenly coiled in a direction which, for a considerable distance, can be diagnosed as right handed. Of the two focal levels required for determination of direction the lower, Fig. b, is the more distinct but in both views the direction of slope of the gyres is unequivocal and is indicated by the angle at which the arrows have been drawn in Figs 9a and b.

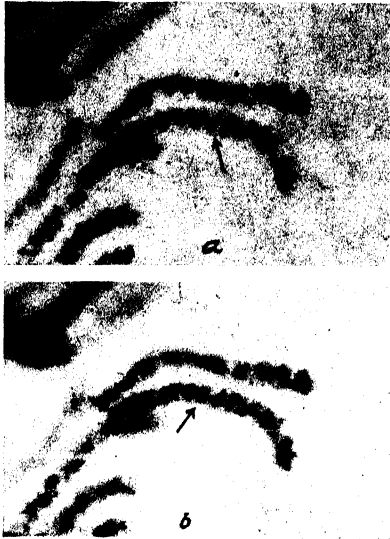


Fig. 9. Detail of chromosome 1. High power U.V.  $\times 4000$ . Two focal levels  $0.4 \mu$  apart. The lower chromatid alone interpretable, with about 14 gyres of a right handed spiral. (Series 102 exposures 3a, 4b).

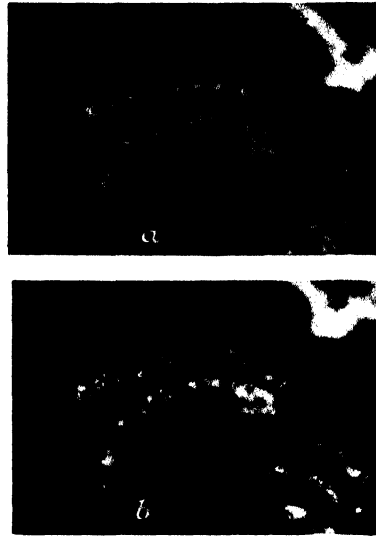


Fig. 10. The same as the preceding but negative print and therefore inverted.

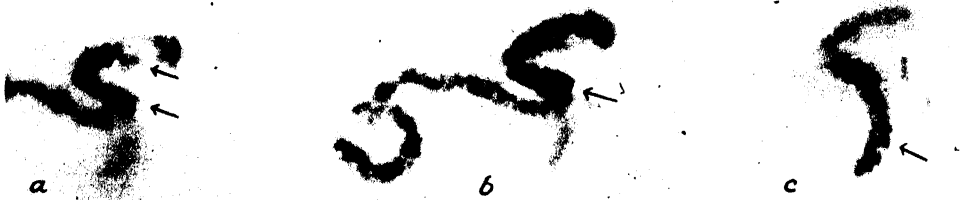


Fig. 11. Detail of chromosome 2 to show approximate number of gyres in the same chromosome as that of Fig. 4. The long arm (Fig. c) has ten gyres, the short arm (Fig. a and b) has four. High power U.V. photograph  $\times 4000$ . (Series 102 exposures 2a, 3b, 9a).

Chromosome 2 (Fig. 11) is less perfect than the preceding and direction of coiling can at no point be determined in it. The number of gyres, however, can be determined fairly clearly in the long arm (Fig. c) and approximately in the short arm, the two chromatids of which are contained in Figs a and b. This chromosome is of interest because it is identifiable by the relative length of the short arm as the same individual as that already described on p. 574 and in Fig. 4. In comparison with Fig. 4, however, the gyres are very nearly twice as numerous, there being 10 instead of 5 in the long arm and 4-5 instead of 3 in the short. This represents a total of 14-15 gyres in the whole chromosome instead of the 8 recorded in Fig. 4.

That all the other chromosomes present have numbers of gyres of the same order is perhaps demonstrated by Fig. 12 which shows an enlarged view of a considerable portion of the left hand nucleus of Fig. 8, some additional details of which appear in Figs 13, 14 and 15.

References p. 584.





Fig. 12. Enlarged view of part of the left hand nucleus of Fig. 8. High power U.V. photograph  $\times 4000$ . (Series 102 exposure 11a).

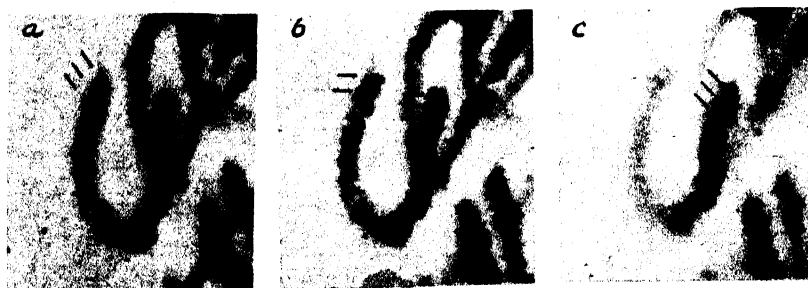


Fig. 13. Three focal levels  $0.4 \mu$  apart to show direction of coiling at the distal ends of the two chromatids of chromosome 3. Ink lines mark the slope of the gyres. Fig. a is the top-most focus. Direction is opposite in the two chromatids.  $\times 4000$  (Series 102 exposures 14b, 11a, 9b).

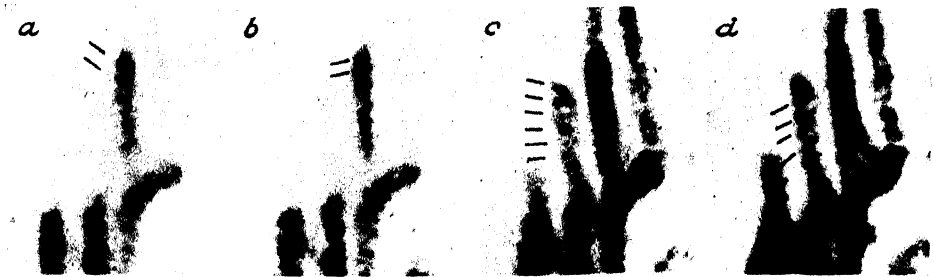


Fig. 14. Four focal levels  $0.4 \mu$  apart to show direction of coiling in the distal ends on the two chromatids of chromosome 4. Ink lines mark the slope of the gyres. Fig. a is the uppermost focus, the rest serially. Direction is similar in the two chromatids.  $\times 4000$ . (Series 102 exposures 7b, 9b, 11b, 13b).

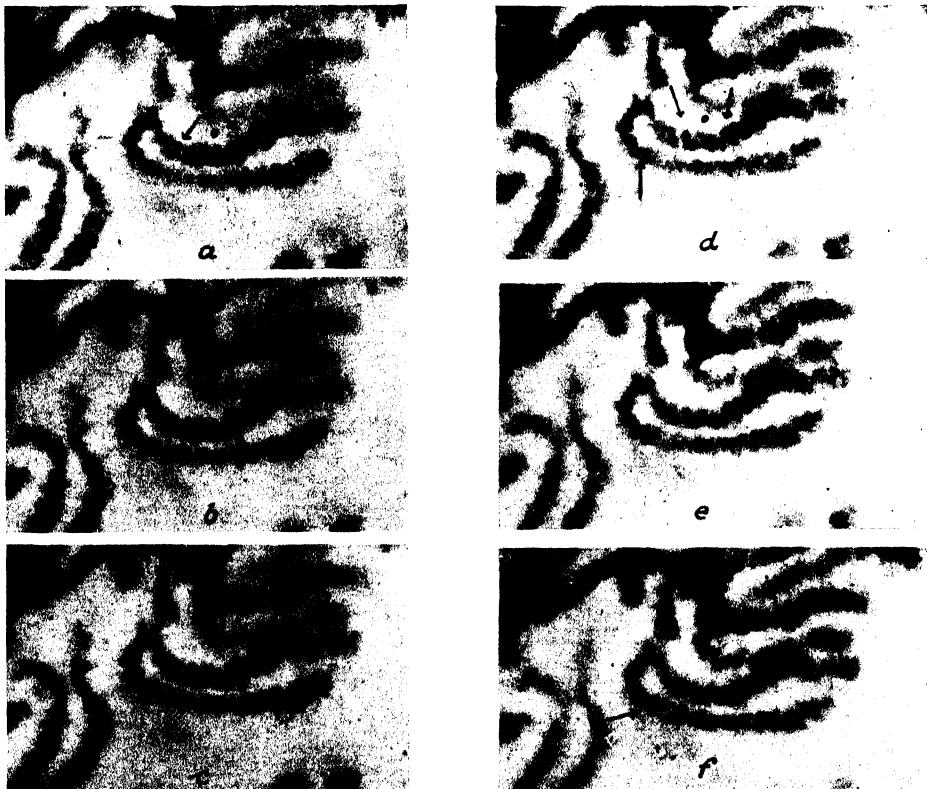


Fig. 15. Details of chromosome 5. U.V.  $\times 4000$ , six focal levels  $0.1 \mu$  apart Fig. a the highest Fig. f the lowest. The upper chromatid contains two changes of direction, the lower chromatid is uninterpretable except for two gyres at the centromere end. (Series 102 exposures 6a-9a).

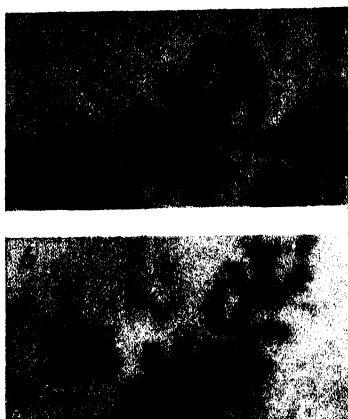


Fig. 16. Late metaphase of the second division in *Osmunda*, two focal levels of parts of one cell photographed with visual light  $\times 2000$  to show reduction of number of gyres per chromosome. Detail of one chromosome visible in Fig. 17.



Fig. 17. Detail of one chromosome from field of Fig. 16b high power U.V. photograph  $\times 4000$ . The blurred outline is due to an opacity of the cytoplasm produced unintentionally by treatment with clove oil. The reduced number of gyres and the increased diameter of the coil in comparison with the earlier state is nevertheless visible. (Series 66 exposure 4a).

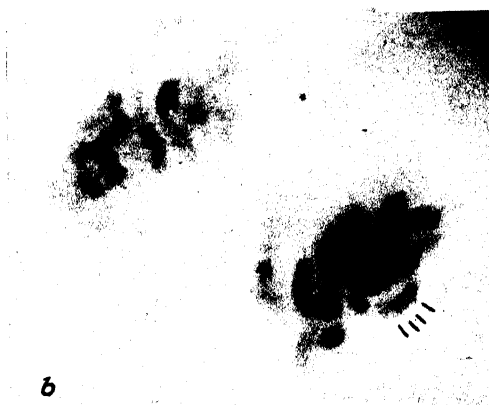
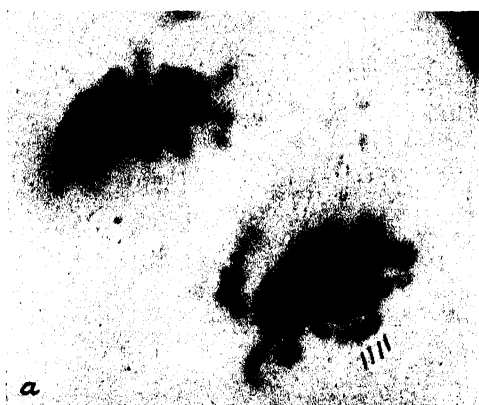


Fig. 18. Two focal levels through sister nuclei at late metaphase of the second meiotic division in *Osmunda* to show reduced number of gyres. Visual light photographs  $\times 2000$ .

Figs 13 and 14 are inserted primarily for further details of direction of coiling. In Fig. 13 three focal levels through chromosome 3 are represented from which direction can be determined in the last two gyres of both chromatids. In the left hand chromatid (Figs 13 a and b) these coils are left-handed but in the right hand chromatid (Figs 13 b and c) they are right-handed; these two chromatids are therefore coiled in *opposite* directions. In Fig. 14, chromosome 4 shows exactly the inverse condition; the distal portions of the two chromatids are *similarly* coiled, namely in a left-handed direction.

That changes of direction can occur along the length of a chromosome can be shown in *Osmunda* no less certainly than in *Tradescantia* although the best single example from the point of view of reproducible clarity belongs to a cell at a slightly

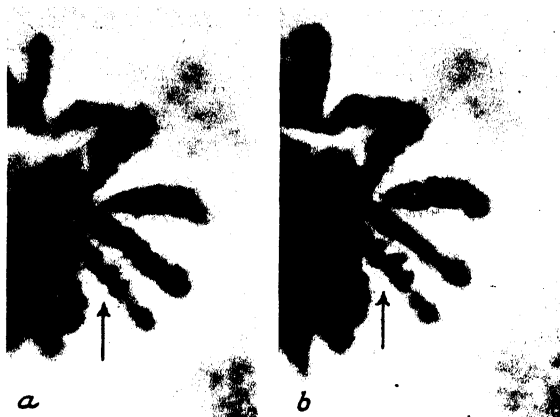


Fig. 19. Two focal levels  $0.4 \mu$  apart through one of the nuclei of the preceding specimen, U.V. photograph  $\times 4000$ . A change of direction very distinctly shown in the chromosome marked by the arrow, especially in the lower focal level (Fig. b). (Series 67 exposures 4a, 5b).

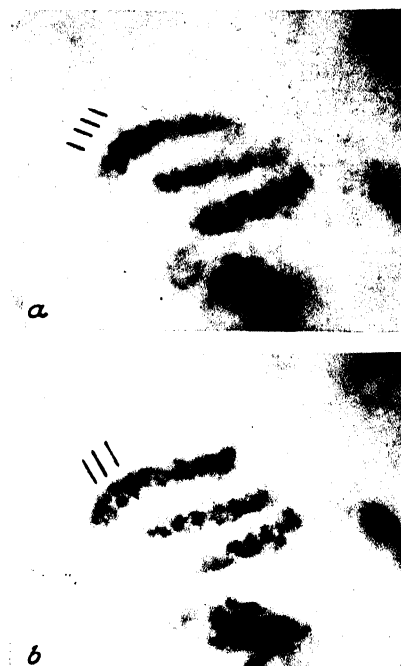


Fig. 20. Anaphase chromosomes from the same preparation as Fig. 17 with gyres of a right-handed spiral visible opposite the ink lines. U.V. photograph  $\times 4000$ . (Series 72 exposures 11a, 12b).

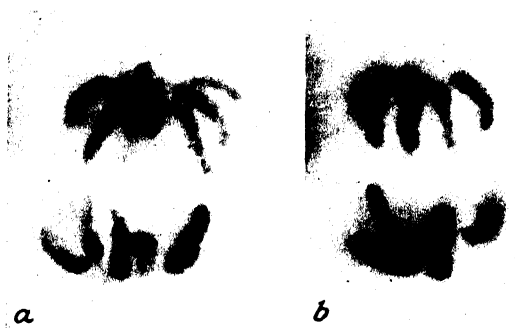


Fig. 21. Anaphase at the same stage as the preceding in another preparation. Two focal levels photographed with visual light  $\times 2000$  to show reduced number of gyres as in Fig. 4 and Fig. 20.

later stage than the one under discussion. It may, however, be helpful to look at the marked chromatid of Fig. 19 and to compare it with the marked chromatid of Figs 7a and b. In both cases the dead point of a change of direction appears in one focal level and the intervening lines in the other; both levels being of course required for full demonstration of the change. In the *Tradescantia* chromosome it so happens that the view containing the dead point is the upper focal level (Fig. 7a). In the *Osmunda* chromosome the comparable view is the lower focal level (Fig. 19b), the lines joining the dead point to the gyres on the two sides of it being visible in Fig. 19a.

Comparable evidence of changes of direction in the cell of Fig. 8 is contained in Fig. 15 although the photographs are more difficult to reproduce. The sequence of photographs in this case represents differences of focal level of  $0.1 \mu$  and as before the significant directions are indicated by arrows drawn parallel to the slope of the gyres. The lower chromatid is uninterpretable except for two gyres near the centromere which are coiled in a right-handed direction (Fig. 15 d-f). The upper chromatid can, however, be analysed for almost its entire length, at least in the original prints. At the centromere

end (Figs 15 a and d) it is also right-handed but about a third of the way down the chromosome direction changes and the middle region of about four gyres is left-handed after which direction changes again. The distal end of this chromatid cannot be clearly determined but the total number of gyres in its whole length is again of the order of 14.

#### THE MINOR SPIRAL AT LATE METAPHASE AND ANAPHASE

Late metaphase is represented here by two cells both of which were obtained rather early in the work before great experience had been gained with the ultraviolet microscope. Both were studied in a preliminary way with visual light, some photographs being reproduced in Figs 16a and b and 18a and b. In both cases the transfer to a quartz slide was made without mishap but subsequent photography was somewhat marred by technical mistakes which were later avoided. In particular the use of clove oil as a medium for preliminary observation with visual light though successful for that purpose as Fig. 16 will illustrate has a deplorable effect on subsequent transparency to ultraviolet and the mistiness of Fig. 17 caused by the opacity of the cytoplasm was wholly due to this treatment.

Owing to these and other defects the ultraviolet pictures (Figs 17 and 19) of late metaphase are less informative than would otherwise have been the case although, in default of better, they have some value for purposes of comparison with the earlier stage. The visual light photographs (Figs 16 and 18) are, however, more extensively reproduced since, for many purposes they are the more informative. In particular the demonstration of the number of gyres per chromosome is best obtained from these photographs. This number, as may be seen in several places in Figs 16 and 18 is not more than 8 per chromatid and in some chromosomes may be slightly less. In the ultraviolet photographs, even that of Fig. 17, this number of gyres is faintly discernible although these photographs are of greater interest for the comparison they give of the diameter of the spiral at late metaphase; in contrast to the earlier stage of Fig. 15, etc., it is markedly wider.

The anaphase condition is represented in visual light by Fig. 21 and in ultraviolet by Fig. 20. The two views of one cell contained in Fig. 21 compare directly with the isolated chromosome of Fig. 4 with the addition that the chromosomes in Fig. 20 are seen in position at the poles. Fig. 21 on the other hand is the only ultraviolet series yet obtained of this stage. It is from another cell in the same preparation as that of Figs 18 and 19 and although far from perfect it is just possible to determine direction of coiling in the distal end of the topmost chromosome and the number of gyres (approximately 8) in the lowermost chromosome. The diameter in all three of these chromosomes contrasts strikingly with the much narrower condition of the spiral in Fig. 15, etc.

#### DISCUSSION

It would therefore seem that the gyres of spiral structure are not constant through the whole of a division but that they are reduced in number and increased in diameter as the division proceeds. At first sight this is a very peculiar type of development for which a causal mechanism is by no means self evident, yet it is a phenomenon which has been described by other workers, notably by SWANSON (1942, 1943) for the first meiotic division and the pollen tube mitosis in *Tradescantia*, and also by SPARROW (1942) for

the pollen grain mitosis of *Trillium*. Its detection now in the second meiotic division of *Osmunda* suggests that it is a general and perhaps an essential feature of the spiralization cycle over a very large part of the plant kingdom.

It is not profitable at this stage to enquire fully into the interpretation of these facts since it is hoped to amplify them shortly in several directions. One special aspect of the problem should, however, perhaps be given brief mention to avoid the risk of profitless speculation; this is the question of chromonema length. In earlier communications (especially MANTON, 1939 and 1945) some fairly precise numerical comparisons of chromonema length were made between a limited number of selected stages of mitosis and meiosis in *Osmunda*. From this it was shown that very considerable changes of length occur during the prophases of the first meiotic division, of a kind which could not be detected in the course of a somatic division. These need not be further discussed here since the present communication does not specifically deal with either of the particular divisions referred to; the previous observation that the chromonema at leptotene or early pachytene is four times as long as it appears to be at the fully spiralized stages of late metaphase or anaphase of either meiotic division is therefore unaffected. When however the comparison is extended to include the early stages of the second meiotic division it might be asked whether, for this division, some modification of the previous position might be required.

Without attempting a final answer to this question at the present stage, it may nevertheless be said at once that such evidence as is available is negative. The accurate measurement of chromosome diameter, which is essential for calculation of chromonema length in the fully spiralized condition, is impossible with the particular techniques used here since these are known to introduce artificial changes which cannot be precisely allowed for. Rough comparisons however, which can for example be made between any of the Figs 9-15 and Figs 17, 19, or 20 show that as gyre number is approximately halved the diameter of the spiral is approximately doubled, indicating either *no* change in chromonema length as gyres are eliminated or at most only a slight shrinkage, too small to be registered with the existing methods and certainly less than 50%. It seems therefore necessary to conclude not only that there is so far no conflict between the new data and previous conclusions for this organisms but also that some physical property other than changes of chromonema length must be primarily involved in the causal mechanism of gyre elimination.

#### SUMMARY

A reduction of the number of gyres per chromosome has been demonstrated to occur during the course of the second meiotic division in *Osmunda*, from approximately 14 at early metaphase to approximately 8 at late metaphase and anaphase.

The diameter of the coil increases as the number of gyres is reduced which suggests that large changes of chromonema length are not involved.

Direction of coiling has been studied in a preliminary way as a necessary part of the demonstration of the reality of the spiral at the various stages. Right-handed and left-handed directions have both been found and corresponding parts of attached chromatids can be coiled either in similar or in opposite directions. Changes of direction can occur more than once along a chromosome arm.

#### RÉSUMÉ

On a démontré qu'il se produit une diminution du nombre des cercles par chromosome pendant la durée de la seconde division méiotique dans *Osmunda*. Ce nombre passe d'environ 14 au début de la

métaphase à environ 8 à la fin de la métaphase et à l'anaphase. Le diamètre de l'enroulement augmente lorsque le nombre de cercles diminue, ce qui permet de penser qu'il ne se produit pas de grandes variations dans la longueur des chromonèmes. La direction de l'enroulement a été étudiée d'une manière préliminaire comme une phase nécessaire de la démonstration de la réalité de la spirale aux différents stades. On a trouvé des enroulements à droite et des enroulements à gauche, et des parties correspondantes de chromatides liés peuvent être enroulées, soit dans une direction similaire, soit dans une direction opposée. Des changements de direction peuvent se produire plus d'une fois le long d'une branche de chromosome.

#### ZUSAMMENFASSUNG

Es wurde bewiesen, dass während der zweiten meiotischen Teilung in *Osmunda* die Anzahl der Windungen per Chromosom abnimmt, und zwar von ungefähr 14 zu Beginn der Anaphase bis etwa 8 am Ende der Metaphase und während der Anaphase.

Der Durchmesser des Knäuels nimmt zu, während die Zahl der Windungen abnimmt, was darauf schliessen lässt, dass die Chromonemalänge nicht stark verändert wird.

Eine vorläufige Untersuchung der Wicklungsrichtung wurde unternommen, da sie einen notwendigen Teil des Beweises zur Existenz von Spiralen in den verschiedenen Stadien bildet. Es wurden sowohl Wicklungen nach rechts wie nach links beobachtet und einander entsprechende Teile von zusammenhängenden Chromatiden können entweder gleichsinnig oder entgegengesetzt gewickelt sein. Entlang einem Chromosomarm kann mehr als einmal Richtungswechsel eintreten.

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## THE FINE STRUCTURE OF THE WALL OF THE CONIFER TRACHEID

## IV. DIMENSIONAL RELATIONSHIPS IN THE OUTER LAYER OF THE SECONDARY WALL

by

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## INTRODUCTION

It is now known (BAILEY AND VESTAL, 1937, WARDROP AND PRESTON, 1947) that the secondary cell wall of conifer tracheids is organized in a series of three coaxial micellar spirals such that the outer and inner spirals are flatter than the central micellar spiral separating them. The latter has been the subject of detailed investigation in previous papers of this series (PRESTON, 1946, 1947, 1948) particularly as regards the relation of its organization to cell dimensions. In earlier work (PRESTON, 1934) use was made of the observation by SANIO (1872), supplemented by the subsequent investigations of BAILEY (1920), that the tracheid length of successive annual rings of a conifer stem gradually increases during the first thirty to fifty years of the growth of the stem, after which it remains more or less constant. This led to the establishment of a connection between the average tracheid length in one annual ring and the cotangent of the average spiral angle of the form

$$L = K \cot \Theta$$

where  $K$  is a constant, and  $\Theta$  is the angle between the micellar winding and the longitudinal cell axis. For reasons given in the last paper in this series, these observations were recently extended to cover individual tracheids within any one annual ring, when it was found that tracheid length,  $L$ , breadth,  $B$ , and spiral angle,  $\Theta$ , were related in the form

$$L = M + N (B^2 \operatorname{cosec}^2 \Theta - 1)^{1/2}$$

This implies that for standard breadth the relation takes the form

$$L = K_0 + K \cot \Theta_{(B=1.00)}$$

The earlier relation, in the lack of any sure evidence of the heterogeneity of the tracheid wall, was taken to apply to the whole wall thickness. It is now known that all three relations apply only to the central spiral layer.

With such a relation demonstrated for the central layer, it becomes of interest to know if a similar relation is applicable to the flatter micellar spiral of the outer and inner

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layers. If this were so, then it would mean that the entire organization of the secondary wall is conditioned by the dimensions of the cell. The present paper is concerned with an investigation, from this point of view, of the conditions in the simpler case of the outer layer. It seems reasonable, in view of the close resemblance of the birefringence in the inner and outer layers, that the inner layer will not differ essentially from the outer in this respect.

#### METHODS AND RESULTS

##### *A. Measurement of micellar orientation in the outer layer*

The method used previously (WARDROP AND PRESTON, 1947, 1949) for determining micellar orientation in this layer was considered too laborious and difficult to be contemplated as a routine method. Advantage was therefore taken of the fact that the simple measurement of birefringence in transverse section of conifer tracheids of increasing length (from successive annual rings) would, provided the intrinsic double refraction of the wall complex remained constant, given an indication of changing micellar orientation in these cells. Thus a decreasing birefringence of this layer in cells from successive annual rings would indicate a spiral in the layer becoming steeper with increasing cell length. It is a disadvantage of such a method that no indication is given of the absolute values of the micellar orientation in any given case, but it would indicate any trend. In point of fact, this difficulty can be surmounted to some extent, as will appear later.

The material examined consisted of a transverse disc taken from the stem of each of the following species:

*Pinus radiata* D. Don,  
*Pseudotsuga douglasii* C. Carr.

Thin transverse sections of the late wood of successive annual rings were cut from each disc, and after dehydration were mounted in canada balsam. The phase difference for each ring was determined using a de Senarmont compensator (AMBRONN AND FREY, 1926) and the birefringence calculated using the section thickness, as described previously (PRESTON, 1946). An average of the values of 20 cells was taken in each case and the results are presented in Tables I and II and Figs 1 and 2.

##### *B. Measurement of cell length*

Small pieces of the late wood were selected from each annual ring, from the same blocks as were sectioned for birefringence measurements, and used for determinations of tracheid length. The specimens were macerated by delignification with chlorine water followed by warming in 0.025% sodium hydroxide (COHEN AND DADSWELL, 1939). A suspension of the tracheids so isolated was allowed to flow over a slide covered with albumen fixative which was then dried in an air oven, placed for five minutes in a 1% aqueous solution of congo red, dehydrated in alcohol, cleared in xylol, and mounted in canada balsam. The slide was then placed in a photographic enlarger and the image of the tracheids was projected at known magnification. The outlines of fifty tracheids from each specimen were then drawn on paper and the lengths of the tracheids were subsequently measured. The results are again presented in Tables I and II and in Figs 1 and 2.

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### C. X-ray Examination

While the X-ray diagram of conifer wood gives no indication of the micellar orientation in the outer layers of the constituent tracheids, change in orientation of the central layer can be followed by this means. It was thought desirable, as a complement to the optical determinations on the outer layer, to examine also by an X-ray method the organization of the corresponding central layer. For this purpose, X-ray diagrams were taken of tangential sections about  $\frac{1}{2}$  mm thick from corresponding regions of the specimens. The spread of the equatorial arcs was determined visually and the spiral angle calculated (see ASTBURY, PRESTON, AND RANGANATHAN, 1949) as a rough check that the relation between orientation in the central layer and cell dimensions was, in the present material, of the type already established for other species. The results are presented in Tables I and II, Figs 1 and 2.

### D. Treatment of data

1. *Calculation of relative micellar orientation from birefringence measurements.* As already stated, the values of the birefringence in Tables I and II cannot be used to calculate the exact values of the micellar orientation in the outer layer of the secondary wall, unless the maximum birefringence of this layer is determined in some way such as that described in a previous paper (WARDROP AND PRESTON, 1947). There are, however, two ways in which a rough approximation can be reached. Either it can be assumed that the intrinsic birefringence already determined for *Picea* applies also to the present material, or the assumption may be made that the maximum birefringence observed in transverse section in the present investigation corresponds to transverse orientation. Since in point of fact the maximum birefringence observed here is actually somewhat greater than the intrinsic birefringence determined for *Picea*, the former method is inapplicable. A maximum birefringence of 0.013 in the case of *Pseudotsuga* (Table I) and of 0.025 with *Pinus* (Table II) were therefore taken as representing transverse orientation. Since the micelles are almost certainly never entirely transverse in these cases, the values obtained for the inclination of the micelles to the longitudinal axis thus determined represent maximum values, so that the inclination is actually steeper than that calculated. Nevertheless such data would indicate the trend of the change in micellar orientation in the outer layer with increasing cell length and does give a clearer mental picture of the point at issue.

The calculation of the relative values of  $\Theta$  can be carried out as follows. If  $n_y$  and  $n_a$  are taken as the major and minor axes of the index ellipsoid of cellulose so that the maximum birefringence is  $n_y - n_a$ , and  $n_{y'} - n_a$  is the birefringence of the outer layer in transverse section when the micelles are inclined at an angle  $\Theta$  to the longitudinal cell axis, then it follows that

$$\frac{(n_{y'})^2 \cos^2 \Theta}{(n_a)^2} + \frac{(n_{y'})^2 (1 - \cos^2 \Theta)}{(n_y)^2} = 1$$

whence,

$$\cos \Theta = \frac{n_a}{n_{y'}} \left\{ \frac{(n_y - n_{y'}) (n_y + n_{y'})}{(n_y - n_a) (n_y + n_a)} \right\}^{1/2}$$

2. *The relation of micellar orientation and cell length.* It will be clear from Tables I and II that the birefringences in transverse section do, in fact, decrease markedly as tracheid length increases. This makes it reasonable to determine whether the data can

TABLE I  
ORIENTATION AND CELL LENGTH IN THE SECONDARY CELL WALL LAYERS OF *Pseudotsuga douglasii*

Annual Ring	Tracheid Length* (mm)	Birefringence of outer layer in Transverse section**	Relative spiral angle outer layer (degrees)	Spiral Angle Central (degrees)
1	2.0	0.006(?)	43	40
3	2.4	0.013	90	29
5	2.6	0.008	52	26
7	3.3	0.009	56	25
9	3.1	0.007	47	20
11	3.3	0.007	47	19
13	3.3	0.007	47	16
15	3.7	0.004	34	13
17	3.9	0.005	39	13
19	3.7	0.006	43	14

\* Average of 50 measurements

\*\* Average of 20 measurements

TABLE II  
ORIENTATION AND CELL LENGTH IN THE SECONDARY CELL WALL LAYERS OF *Pinus radiata*

Annual Ring	Tracheid Length* (mm)	Birefringence of outer layer in Transverse section**	Relative spiral angle outer layer (degrees)	Spiral Angle Central (degrees)
1	1.7	0.022	71	38
2	—	0.025	—	—
3	2.0	0.025	90	42
4	—	0.024	—	—
6	2.4	0.024	79	38
8	2.9	0.023	74	26
10	2.9	0.020	64	20
12	3.2	0.020	64	27
15	3.2	0.018	58	20

\* Average of 50 measurements

\*\* Average of 20 measurements

be fitted to a relation between micellar orientation and cell length of the type described above for the central layer. This was done by using the relative values of  $\theta$  for the outer layer and determining the line of closest fit for the data of  $L$  and  $\cot \theta$ . This procedure was also applied to the values of  $L$  and the actual values of  $\theta$  for the central layer, for which the relation  $L = K_0 + K_1 \cot \theta$  was originally developed. In both cases the spiral clearly becomes steeper the longer the tracheid. With the outer layer the spread of the observations is greater than in the central layer but whether this difference is due to experimental error or to other factors cannot be decided. The line of closest fit was found by the method of least squares (YULE AND KENDALL, 1937) and the resulting equations for the data in Tables I and II, and presented in Figs 3 and 4 are as follows:

*Pseudotsuga douglasii*

Central Layer . . . . .  $L = 1.60 + 0.52 \cot \theta$

Outer Layer . . . . .  $L = 2.34 + 0.86 \cot \theta$

*Pinus radiata*

Central Layer . . . . .  $L = 0.57 + 1.09 \cot \theta$

Outer Layer . . . . .  $L = 1.48 + 3.28 \cot \theta$

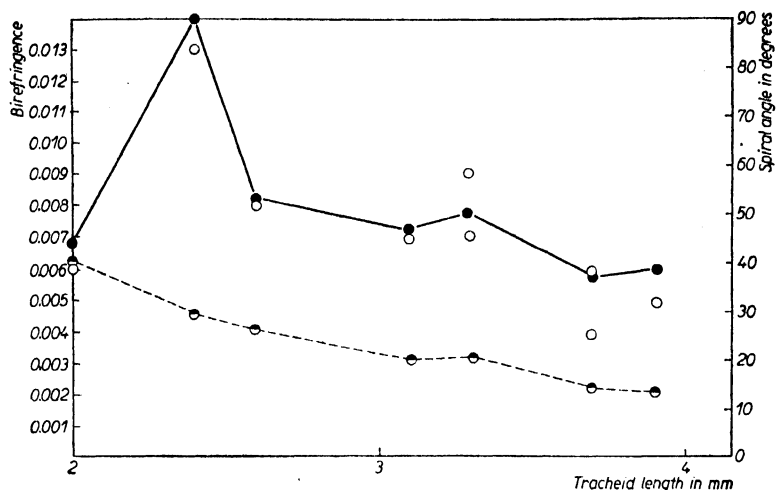


Fig. 1. The relation between birefringence and relative spiral angle in the outer layer, and the observed spiral angle in the central layer, for *Pseudotsuga*. ○ Birefringence in transverse section of outer layer; ● Relative spiral angle in outer layer; ● Spiral angle in central layer. Note that the birefringences are plotted individually, while for simplicity of drawing the angles calculated for a tracheid length where two angles have been determined are the averages of these angles.

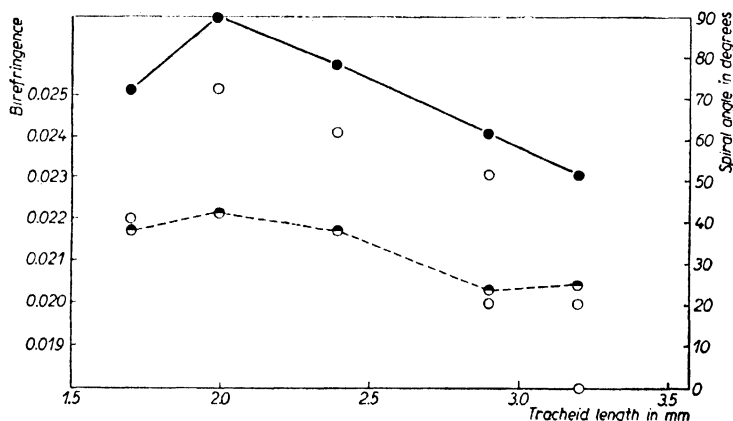


Fig. 2. Data as in Fig. 1 for *Pinus*

#### DISCUSSION

From the above data it is clear that the observed changes in birefringence with increase in cell length are consistent with the view that the spiral micellar orientation in the outer layer, like that in the central layer, becomes steeper as cell length increases. It can thus be said that the entire organization of the secondary wall layer is governed by the length of the cell.

These results also possess considerable interest in relation to the work of BAILEY AND VESTAL (1937), upon the cell wall organization of conifer tracheids. These investi-

gators have claimed that conifer tracheids can be grouped, according to the organization of their cell wall, into four types such that the micelles are:

1. in the central layer parallel to the major cell axis and in the outer layer perpendicular to it;
2. in the central layer parallel to the major cell axis and in the outer layer possess a spiral orientation;
3. in the central layer spirally orientated with respect to the major cell axis and in the outer layer perpendicular to it, and
4. in both the central and outer layers possess a spiral orientation with respect to the major axis of the cell.

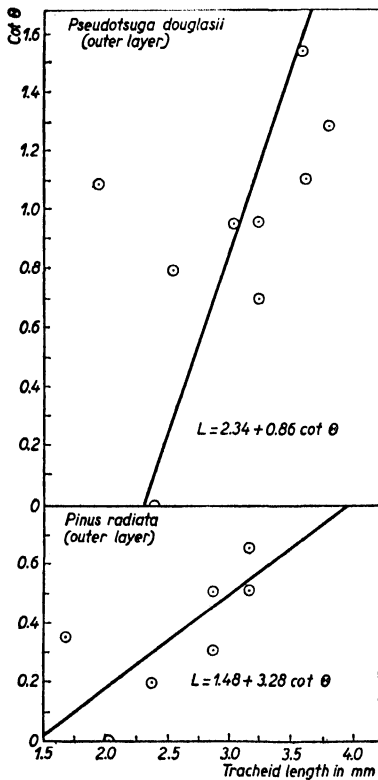


Fig. 3. The relation between tracheid length and  $\cot \theta$  for *Pseudotsuga*, outer layer and *Pinus*, outer layer

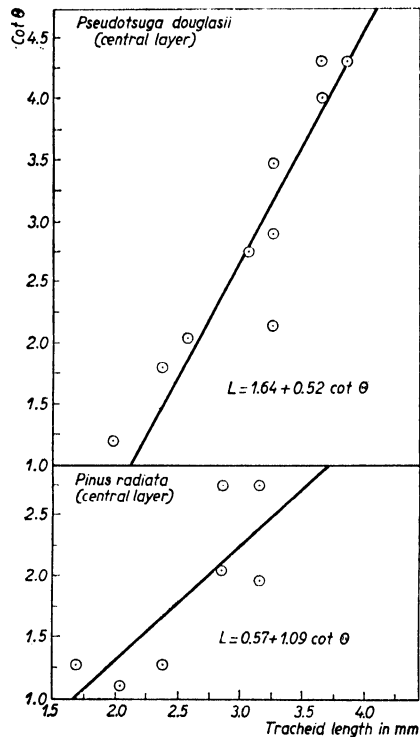


Fig. 4. The relation between tracheid length and  $\cot \theta$  for the central layer of *Pseudotsuga* and *Pinus*

These conclusions were based upon the observed direction of growth of iodine crystals in swollen cell walls and it is not, in any case, clear what they mean in terms of the structure of the untreated wall. The point which should be made here, however, is that the above groups (2), (4) and (3) simply correspond to the expected organization of tracheids from outer, intermediate and inner annual rings respectively between which all grades of organization may be found. Thus the "types" described by these workers are but variations of the general pattern of micellar organization in conifer tracheids.

As for type (1) above there is no evidence in the present data that such an organization exists although it was almost realized in the wood fibres of *Nothofagus cunninghamii* (WARDROP AND PRESTON, 1947). It is to be suggested that perhaps type (1) above may represent a tracheid of type (4) which was distorted in a manner such that the micellar spiral of the outer layer became flatter and the central spiral steeper. In any case, because of the technique employed the recognition of this type of organization is, of necessity, suspect.

The fact that the micellar orientation of the outer layer of the secondary wall becomes steeper with increasing cell length also bears upon the problem as to why this layer does not contribute any detectable pattern to the X-ray diagram (PRESTON, 1946). Thus if the inclination in the outer layer is, say, only  $45^\circ$ , the contribution of this layer would appear as a "tail" to the equatorial arcs from the central layer particularly since the micelles of the outer layer would seem to possess some considerable angular dispersion (WARDROP AND PRESTON, 1947, 1949) and would have their contribution to the X-ray diagram further reduced by diffuse scattering from lignin and other amorphous materials present in this layer. Furthermore the appearance of meridional arcs arising from micelles oriented transversely to the major cell axis would be expected to be found only in cells of which the micelles of the central layer were present in a relatively flat spiral. Now, as PRESTON (1946) has shown, a spurious meridional arc arises in the X-ray diagram of the central layer when the micellar spiral is relatively flat, so it would seem that the very conditions under which transversely oriented micelles of the outer layer could be detected by X-rays are also the conditions under which spurious meridional arcs arise, at almost the same position on the X-rays diagram.

It may perhaps further be pointed out that if the spirals of the outer and inner layers of the secondary walls discussed here have the same sign, as they were shown to have in *Picea* sp. (WARDROP AND PRESTON, 1947) then the inter-layer relationship resembles that already demonstrated in *Cladophora* (ASTBURY AND PRESTON, 1940) and in some vessels (PRESTON, 1939) in the sense that as the spiral micellar organization changes the angle between the spirals tends to remain constant. It may be significant that this is in contrast to the primary-secondary wall relationship in the only case which has yet been studied (PRESTON, 1947), where the orientation in the primary wall is apparently quite independent of that in the central layer of the secondary wall.

In conclusion, therefore, the demonstration presented in this paper that the micellar organization in the outer layer of the secondary wall, as well as that in the central layer, is dependent upon cell dimensions, further emphasizes the distinction between primary and secondary walls, since in the former no such relation exists.

#### SUMMARY

A study of the optical properties of the outer layer of the secondary wall of tracheids of *Pseudotsuga douglasii* C. Carr and of *Pinus radiata* D. Don (*Pinus insignis*) shows that the micellar organization of the cellulose component is dependent upon tracheid length. This has been interpreted as a steepening of the micellar spiral with increasing length, and it has been shown that the relation between cell length and the spiral angle is of the same kind as that previously demonstrated in the central layer. Thus the organization of the whole secondary wall is shown to be dependent upon cell length.

#### RÉSUMÉ

L'étude des propriétés optiques de la couche externe de la paroi secondaire des trachéides de *Pseudotsuga douglasii* C. Carr et de *Pinus radiata* D. Don (*Pinus insignis*) montre que l'organisation  
*References p. 592.*

micellaire du constituant cellulosique dépend de la longueur du trachéide. Ceci est probablement dû à ce que la spirale micellaire s'étire lorsque la longueur du trachéide s'accroît; il a été montré que la relation entre la longueur de la cellule et l'angle de la spirale est analogue à celle qui existe dans le cas de la couche centrale. Ainsi l'organisation de toute la paroi secondaire dépend de la longueur de la cellule.

### ZUSAMMENFASSUNG

Die Untersuchung der optischen Eigenschaften der äusseren Schichte der Sekundärwand von Tracheiden von *Pseudotsuga douglasii* C. Carr und *Pinus radiata* D. Don (*Pinus insignis*) zeigt, dass der mizellare Aufbau des Zelluloseanteils von der Länge der Tracheiden abhängt. Dies wurde durch ein Steilerwerden der Mizellenspirale mit wachsender Länge der Tracheiden erklärt. Es wurde gezeigt, dass das Verhältnis zwischen der Zelllänge und dem Spiralwinkel dem früher für die Mittelschichte bewiesenen ähnelt. So hängt also der ganze Aufbau der Sekundärwand von der Zelllänge ab.

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## STUDIES OF ONION ROOT RESPIRATION

I. VELOCITY OF OXYGEN CONSUMPTION IN DIFFERENT SEGMENTS  
OF ROOT AT DIFFERENT TEMPERATURES AS  
A FUNCTION OF PARTIAL PRESSURE OF OXYGEN\*

by

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The relationship between oxygen tension and respiratory rate has been studied for a number of animal and plant species. TANG<sup>1</sup> reviewed the literature up to 1933 and found that hyperbolic curves were usually obtained when rate of oxygen consumption was plotted against pressure of oxygen. Beyond a certain critical pressure respiration was constant. There are three possible explanations for these results: (1) there is a direct mass action dependency on oxygen concentration (pressure) in the kinetics of respiration (KEMPNER<sup>2</sup>); (2) diffusion alone is limiting and the diminished rate of respiration at progressively lower oxygen pressures is due to an increasing core of anaerobiosis in the system (WARBURG<sup>3</sup>, GERARD<sup>4</sup>); and (3) to a combination of (1) and (2). Because diffusion can act as a limiting factor in respiratory rate, it is now standard procedure to cut tissue slices within a maximum thickness so as to eliminate diffusion as a complication in studies on oxidative metabolism. In contrast to this *in vitro* problem, KROGH<sup>5</sup> has calculated that capillary supply in vertebrate organisms is such that diffusion in all probability adequately supplies the body cells with oxygen.

The relationship between oxygen pressure and rate of oxygen consumption in higher plants has been less thoroughly investigated. Many publications are available in which the rate of carbon dioxide evolution has been measured at different oxygen pressures. MACK<sup>6</sup> gives an extensive review of the earlier work of this type and more recently CHOUDHURY<sup>7</sup>, PLATENIUS<sup>8</sup>, and BIALE AND YOUNG<sup>9</sup> have made important contributions. These studies are valuable in arriving at optimum conditions for storage, etc., but offer little evidence of the dynamics of gaseous exchange. The change in rate of production of carbon dioxide at pressures of oxygen lower than air is dependent primarily upon the anaerobic rate compared with the aerobic rate. There seems to be considerable variation in this from one species of plant to another (CHOUDHURY<sup>7</sup>). Thus if the carbon dioxide output were the same both aerobically and anaerobically, respiration, evaluated in this way, would appear to be independent of oxygen pressure. VLAMIS AND DAVIS<sup>10</sup> measured both oxygen consumed and carbon dioxide produced by rice and barley seedlings. Oxygen uptake *vs* oxygen pressure curves were typically hyperbolic

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but at the lowest oxygen pressure investigated, 0.2%, oxygen consumption was only 15% of that in air, while carbon dioxide output was about 50%. In similar studies, TAYLOR<sup>11</sup> found the oxygen consumption of very young wheat and rice seedlings to decrease rapidly with lowered oxygen pressure but carbon dioxide evolution declined under the same conditions for wheat and increased for rice. This would account for the wide range of oxygen pressures at which the minimum rate of respiration, evaluated by carbon dioxide production, occurs in different tissues.

Measurements of the velocity of oxygen consumption in young roots as a function of oxygen pressure have been reported by WANNER<sup>12</sup> AND BERRY<sup>13</sup>. WANNER, using a polarographic technique, concluded that the rate of diffusion of oxygen was the limiting factor in the respiration of roots and that this was dependent upon temperature. BERRY employed the WARBURG manometric procedure and found at 25° C that the limiting pressure of oxygen depended upon the segment of root under investigation. It was further shown that the anaerobic production of carbon dioxide was less than it was aerobically in each of the three segments investigated. A differential rate of respiration for various polar zones of roots had previously been shown to exist for roots of wheat and onion by WANNER<sup>14</sup>, for barley by MACHLIS<sup>15</sup>, for onion by BERRY AND BROCK<sup>16</sup>, and for barley, rape, corn, onion, and bean by PREVOT<sup>17</sup>.

The present investigation was undertaken in order to analyse more completely the relationships between diffusion and temperature in the oxygen consumption of different zones along the axis of onion roots. These data strongly suggest that diffusion becomes limiting at progressively higher oxygen tensions as temperature increases and that the temperature characteristic of respiration assumes a value typical of diffusion when diffusion is predominantly limiting.

#### METHOD

Roots were grown and prepared for respiratory measurements according to the methods described in detail by BERRY<sup>13</sup>. All experiments were carried out in an airconditioned dark room with neon lights made of ruby glass tubing as the only illumination. A small flashlight was used for reading the manometers. The temperature in the room varied between 20–25° C. This was less constant than previously reported<sup>13</sup> because of poorly controlled heating during the winter months. For respiratory measurements below room temperature, the water bath in which the reaction vessels were immersed was cooled to 20° C by circulating tap water through coils of copper tubing and to reach 15° C ice water was circulated by means of a centrifugal pump.

Two-day old roots were used exclusively in these experiments and the number of segments added to each manometer flask varied with the temperature at which gaseous exchange was being measured. As many as 130 roots were needed at 15° C to give pressure changes comparable to 30 or 40 roots at 30° or 35° C. Thus by having essentially the same manometer readings at the different temperatures, a possible source of variable error is eliminated. The duration of each run was one or two hours.

Different partial pressures of oxygen were obtained by the evacuation method described by UMBREIT, BURRIS, AND STAUFFER<sup>18</sup>, using commercial tank oxygen and nitrogen without further purification. Calculations show that this yields final values accurate to approximately 0.1%.

Rates of respiration are given as cubic millimeters of oxygen consumed per root segment per hour. These values may be converted to  $Q_{O_2}$  per gram wet weight or per gram dry weight by multiplication with the conversion factors shown in Table I. The average weights were determined by a series of weighings on a Kuhlmann microbalance.

All experiments were carried out with root segments cut 5, 10, or 15 mm above the tip. The respiratory rate for each of the two zones, 5–10 mm, and 10–15 mm above the tip could then be calculated by difference. The apical 5 mm is essentially a zone of mitotic activity, the next zone is one of elongation and differentiation and the third region, 10–15 mm above the tip, consists predominantly of mature cells. In this procedure only one cut is required in arriving at the velocity of oxygen uptake for each of the three zones of root tip. BERRY<sup>13</sup> has shown that the values obtained in this

way differ from those found when each segment is employed directly by amounts that fall within the limits of accuracy of the manometric technique.

TABLE I

Root segment	Conversion factor to change $\mu\text{l O}_2/\text{root}/\text{h}$ to $\text{Q}_{02}$ per g	
	wet weight	dry weight
0-5	501	8520
5-10	548	13940
10-15	591	18640

## RESULTS

1. *Respiratory rate as a function of oxygen tension at 15° C*

Fig. 1 shows the respiratory rate in  $\mu\text{l}$  of oxygen per root per hour plotted against percent of oxygen in the manometer vessel for each of the three zones. Every point is the average of from three to six separate determinations. The maximum probable error occurred with the point at 5% oxygen for the 0-5 mm zone and amounted to slightly less than 8% of the value plotted. Most probable errors fell within the 3-5% range. The curves show that a rapid decrease in the rate of oxygen consumption occurs in each segment when a certain critical oxygen pressure is reached. At pressures higher than this, the respiratory rate is constant regardless of the oxygen tension. For this temperature, breaks in the curves fall at 10% oxygen for both the 5-10 mm and 10-15 mm regions above the tip and at 15% oxygen for the apical 5 mm. Since a low temperature is generally believed to depress metabolism more than it does a physical process such as diffusion (see MOELWYN-HUGHES<sup>19</sup>), one might expect to reduce the importance of diffusion to a minimum when the temperature is low.

Table II (columns 2 and 3) shows the values of carbon dioxide production for the 0-5 mm zone under the same conditions. Both volume of carbon dioxide in  $\mu\text{l}$  evolved per root per hour as well as the values for respiratory

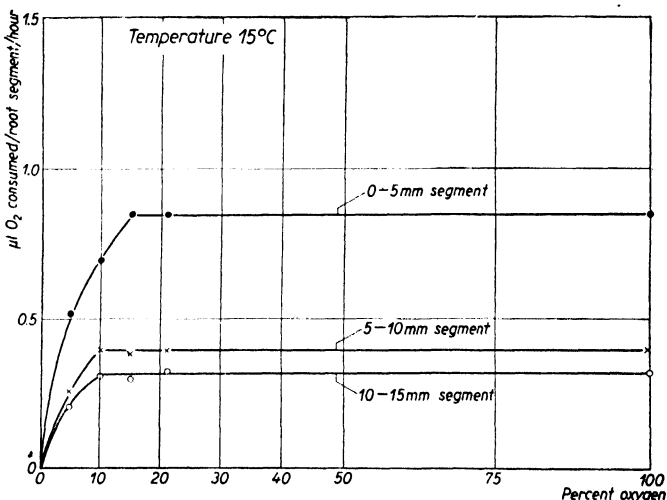


Fig. 1. Oxygen consumed in different root zones as a function of oxygen pressure at 15° C

quotient are given. It can be seen that the total output is reduced at pressures of oxygen below the critical pressure and that fermentation is clearly evident at 5% oxygen. R.Q. values so near unity suggest that essentially pure carbohydrate serves as substrate. Even though there is a reduction in carbon dioxide evolution below the critical pressure (15% oxygen) for this zone of the root, the relative magnitude of reduction is less than that observed for oxygen. Respiratory rate measured by carbon dioxide production would show, however, at this temperature, the same dependency on oxygen pressure as that shown in Fig. 1. Values for carbon dioxide in the 5-10 mm and 10-15 mm segments were found to be quite variable, less so at 15° C than at the higher temperatures. Because of this difficulty, it is believed that little significance could be attached to these results at this time. They clearly indicate at this temperature, however, the same trend as that shown for the most apical 5 mm. The R.Q.'s are about 1.2 at 5% oxygen.

TABLE II  
CO<sub>2</sub> OUTPUT AND RESPIRATORY QUOTIENTS FOR THE 0-5 mm SEGMENT OF ROOT AT DIFFERENT TEMPERATURES AND OXYGEN PRESSURES

(1) Pressure of oxygen	15° C		20° C		30° C		35° C	
	μl/root /hour	(3) R. Q.	μl/root /hour	(5) R. Q.	μl/root /hour	(7) R. Q.	μl/root /hour	(9) R. Q.
5 %	0.60	1.15	0.84	1.38	1.59	2.12	1.90	2.83
10 %	0.69	0.98	1.02	1.13	1.69	1.26	2.51	2.24
15 %	0.86	1.00	1.15	0.99	1.89	1.16	2.51	1.55
21 % (air)	0.86	1.01	1.28	1.04	2.18	1.11	2.86	1.43
40 %							3.40	1.35
45 %					2.85	1.03		
50 %					2.90	1.04	3.27	1.23
100 %	0.88	1.04	1.22	0.97	2.89	1.05	3.18	1.19

## 2. Respiratory rate as a function of oxygen tension at 20° C

Fig. 2 summarizes the data on oxygen consumption for each root zone at 20° C. Each point is the mean of from 4-16 separate determinations and the probable error in all cases is less than 3% of the average value plotted. The critical pressures occur in air for the 0-5 mm segment, at 15% oxygen for the 5-10 mm segment and at 10% oxygen for the 10-15 mm zone. This is an increase for the two more apical segments over the values found at 15° C. Attention is also called to the slight depression in the 10% oxygen value for the 10-15 mm segment. This point is just enough lower to be

within the approximate limit of accuracy of the method. The increase in rate of oxygen consumption with temperature can be seen by comparing the constant levels in Figs 1 and 2. Each zone shows approximately the same relative increment. The values for carbon dioxide at 20° C are given in Table II, columns 4 and 5 for the 0-5 mm zone. The R.Q.'s are again approximately 1.00 except at the two lowest oxygen pressures where fermentation becomes significant. The decrease in volume of carbon dioxide evolved per root per hour as a function of oxygen tension again coincides with that for oxygen consumption but the percentage decrease in respiration based on carbon dioxide values is less than that for oxygen.

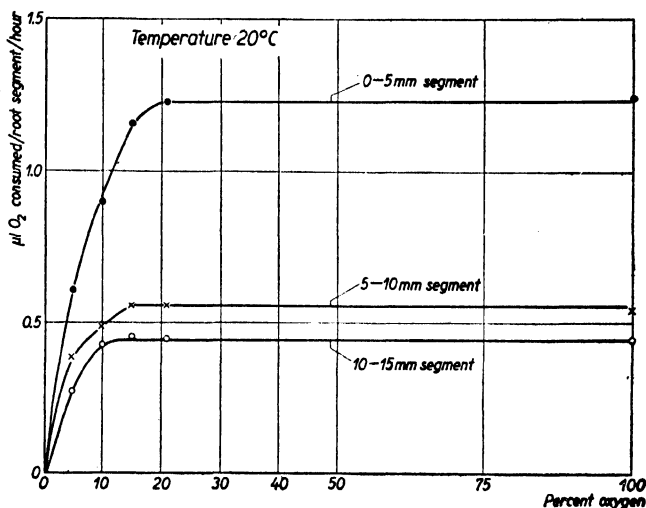


Fig. 2. Oxygen consumed in different root zones as a function of oxygen pressure at 20° C

### 3. Respiratory rate as a function of oxygen tension at 30° C

(The values for 25° C have been reported by BERRY<sup>13</sup>).

The curves in Fig. 3 summarize the results at 30° C. Each point is the average of from three to eight separate determinations and the probable errors range from 5% of

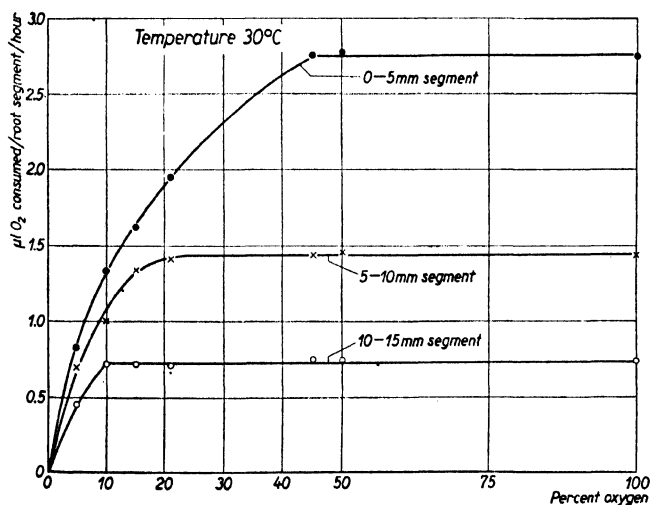


Fig. 3. Oxygen consumed in different root zones as a function of oxygen pressure at 30° C

the value at 45% oxygen in the 0-5 mm zone to 0.1% for the same zone at 100% oxygen. Most fall between 3% and 4%. The critical pressures are 45% oxygen for the 0-5 mm segment, 21% (air) for the 5-10 mm segment and 10% oxygen for the 10-15 mm segment. Large increases in the maximum rate of oxygen consumption are easily seen for each segment as compared to the rates at 15° C and 20° C.

The carbon dioxide values and R.Q.'s are listed in Table II, columns 6 and 7. The parallelism between these results and those for oxygen are again apparent. The relative decrease in carbon dioxide evolved at 5% oxygen compared with that at 100% oxygen is much less than the decrease in oxygen consumption at the

same partial pressures. The R.Q.'s show the progressive increase in anaerobiosis and fermentation that accompanies the diminishing percentage of oxygen.

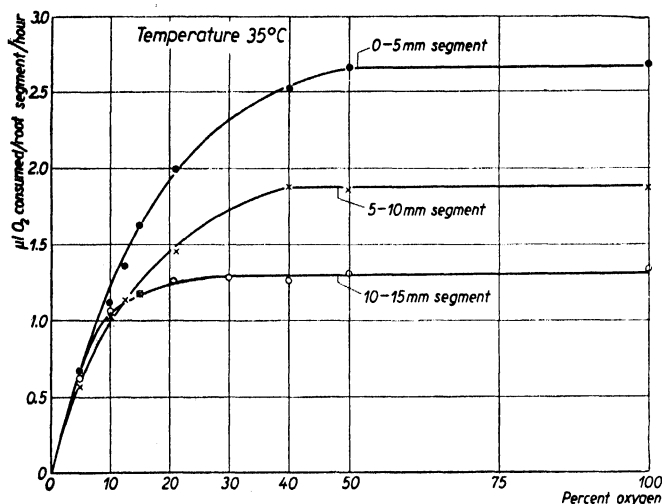


Fig. 4. Oxygen consumed in different root zones as a function of oxygen pressure at 35° C

not accompanied by as high a mean rate of oxygen consumption, calculated on an hourly basis, as that found for 30° C (2.66  $\mu\text{l}/\text{root}/\text{hour}$  versus 2.76  $\mu\text{l}/\text{root}/\text{hour}$ ). However, as Fig. 5 shows, there is a progressive decline in the rate of respiration

with time at 35° C in an atmosphere of pure oxygen. These curves were drawn with each point representing the average value of six separate experiments. A decrease in rate with time was observed only at this temperature for the periods of measurement used in these experiments. When the oxygen consumed during the first quarter hour is used to calculate the hourly rate of respiration for this segment, an uptake of 3.06  $\mu\text{l}/\text{root}/\text{hour}$  is found. This is higher than the value at 30° C even though at 35° C an inactivation of enzymes seems to occur. A smaller decline in rate is also to be found in the 5-10 mm segment, but it becomes apparent only after a longer period than that required with the apical 5 mm. The basal segment has a constant rate of oxygen consumption during this period. It has also been found that carbon dioxide evolution

#### 4. Respiratory rate as a function of oxygen tension at 35° C

The results at this temperature are given in Fig. 4. From 3-9 separate determinations were averaged for each point and the largest probable error (6%) occurred with 9 determinations in air for the 5-10 mm segment. The critical pressures are 50% oxygen for the 0-5 mm region of root, 40% oxygen for the 5-10 mm segment and air for the 10-15 mm zone. It should be noted that the higher critical pressure at this temperature for the most apical 5 mm of root is

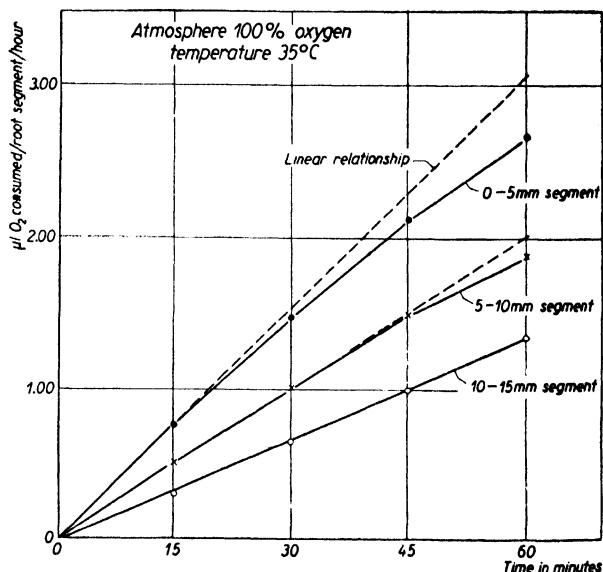


Fig. 5. Oxygen consumed in different root zones as a function of time at 35° C, and in an atmosphere of pure oxygen

diminishes with time in the first segment but it is less than that for oxygen consumption and is virtually absent in the two more basal segments. The constant rates of respiration for these segments show considerable increase over the rates at 30° C (compare Figs 4 and 3) so that they are not strictly comparable with the apex.

Reference to Table II, columns 8 and 9, further brings out the abnormal behaviour of the apex at 35° C. The R.Q. is 1.2 even at oxygen pressures above the critical level and rises steadily to 2.8 at 5% as the percentage of oxygen declines. A greater sensitivity to heat inactivation of those enzymes responsible for the utilization of molecular oxygen rather than the enzymes involved in fermentation might account for the high R.Q. at this temperature in the presence of pure oxygen. This was not at all true for the 10–15 mm segment which had normal R.Q. values down to 15% oxygen. Carbon dioxide production in the apex dropped only at pressures lower than 40% (50% critical pressure) and, as was true for all other temperatures, there was a smaller decrease in rate of output of carbon dioxide at 5% oxygen than was seen for oxygen consumption.

When the rate of oxygen consumption by the different zones of the root at 35° C is converted to  $Q_{O_2}$  by the conversion factors in Table I, very high values are obtained. A comparison with those listed by GODDARD<sup>20</sup> for a variety of animal and plant tissues shows that the meristematic region of the onion root is among the highest. This fact emphasizes the intense energy production that occurs in these cells as well as in those of animal origin.

The similarity of the respiratory rates at which certain pressures of oxygen become limiting is of interest. At three different temperatures air is the critical pressure. This was found for the 0–5 mm zone at 20° C, for the 5–10 mm zone at 30° C, and for the 10–15 mm zone at 35° C. The hourly rate was 1.23, 1.45, and 1.28  $\mu$ l of oxygen per root respectively. It thus appears that for a given pressure of oxygen, only a certain velocity of respiration can be supported, independent of the root zone involved. This is again indicated by the 1.87  $\mu$ l/root/hour rate shown by the 5–10 mm segment at 35° C, where the critical pressure is 40% oxygen and the 1.73  $\mu$ l/root/hour for the 0.5 mm segment at 25° C where calculations show the critical pressure to be about 30% or more oxygen (see BERRY<sup>13</sup>). It also shows the difficulty that might be expected in accurately determining the limiting pressure for those segments with low oxygen uptake. The 10–15 mm segment had the same critical pressure (10%) at 15° C, 20° C, and 30° C, even though the respiratory rate more than doubled over this temperature interval. The accuracy of the 30° C value might be called into question but three separate determinations at 10% oxygen checked with a probable error much less than 1% of the average rate. This was also true for the other two segments under the same conditions. However, 15% oxygen was the critical pressure for the 0–5 mm segment at 15° C with a rate of 0.85  $\mu$ l of oxygen per root per hour and also for the 5–10 mm segment at 20° C with a rate of 0.55  $\mu$ l per root per hour. The rate for the 10–15 mm segment at the same oxygen pressure at 30° C was 0.73  $\mu$ l per root per hour. For more precise determinations, oxygen pressure changes in steps less than 5% should be used with the lower respiratory rates but then one is confronted with the problem of precisely measuring a small but significant change in rate of oxygen consumption.

##### 5. *Temperature characteristics as a function of oxygen pressure*

In spite of the criticisms<sup>21, 22</sup>, aimed at the use of temperature characteristics in elucidating the underlying mechanisms of biological processes, it is believed that by calculating the  $\mu$  value in the Arrhenius equation, the information obtained can be used

in evaluating the results reported above. For this purpose the curves in Fig. 6 should first be examined since these represent the data for each root zone obtained in an

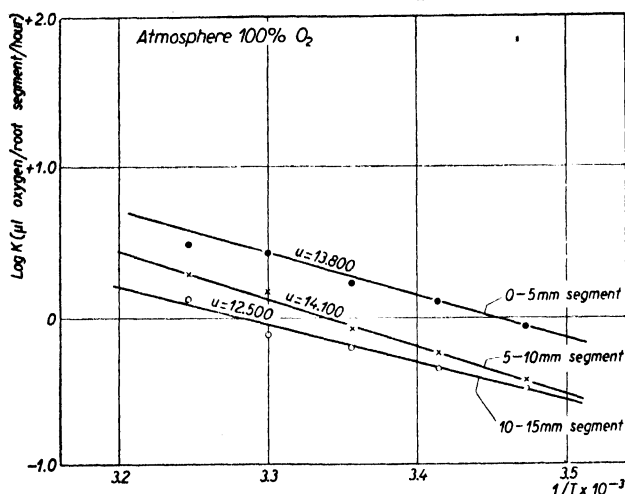


Fig. 6. Log K ( $\mu$ l of oxygen consumed /root segment/ h) plotted against the reciprocal of the absolute temperature, for different root zones in an atmosphere of pure oxygen

atmosphere of 100% oxygen. The process of gaseous diffusion into the locus of change in the root should not be a limiting process in pure oxygen. The  $\mu$  values were calculated from those points falling on the curves and the agreement in the numerical values for the slope of each curve is good for data of this type. The point shown at  $35^\circ\text{C}$  ( $1/T = 3.25 \cdot 10^{-3}$ ) for the 0-5 mm segment is the value derived when the maximum rate ( $3.06 \mu$ l of oxygen per root per hour) was calculated from the initial 15 minute uptake (see Fig. 5) and it falls only slightly below the expected value. All curves were drawn by inspection to give

the best fit for the points. No suggestion of a break in any curve can be seen except between  $30^\circ\text{C}$  and  $35^\circ\text{C}$  in the one for the 0-5 mm segment. If any significance can be attached to these results, it would be to suggest that comparable reactions are proceeding in each of the root zones.

If reference is now made to the curves in Fig. 7, drawn for the rate of oxygen consumption in each root segment at 5% oxygen, a pronounced difference is seen between the one for the 0-5 mm segment and those for the more basal segments of root. The slope ( $\mu$  value) for the apex is now equivalent to 4300 calories per mole, and for the other two curves it is 11300. SIZER<sup>23</sup> gives the temperature characteristic of diffusion as approximately 3000 calories; GLASSTONE, LAIDLER, AND EYRING<sup>24</sup> report values lying between 6600 calories and 3800 calories, depending upon the temperature at which the measurement is made as well as upon the actual substances involved; and MOELWYN-HUGHES<sup>19</sup> states that under conditions where the rate of a heterogeneous reaction is determined by a diffusion

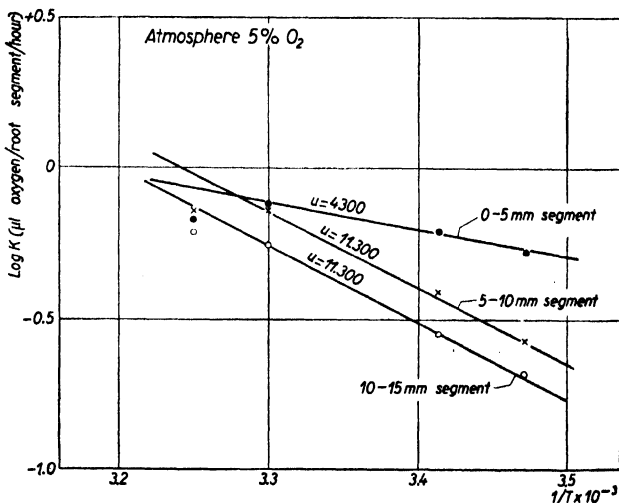
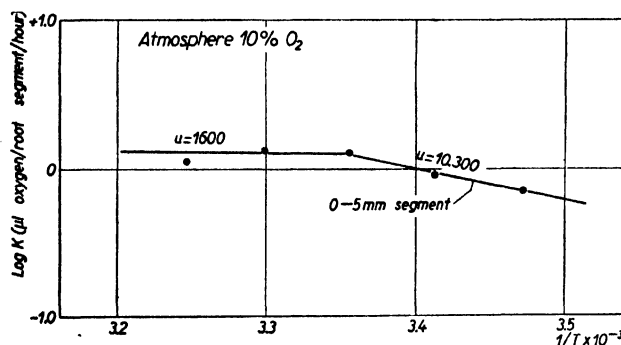
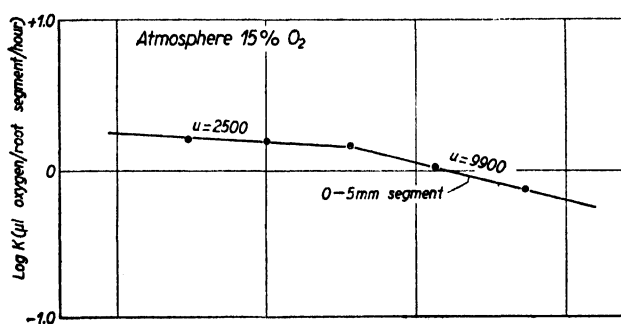
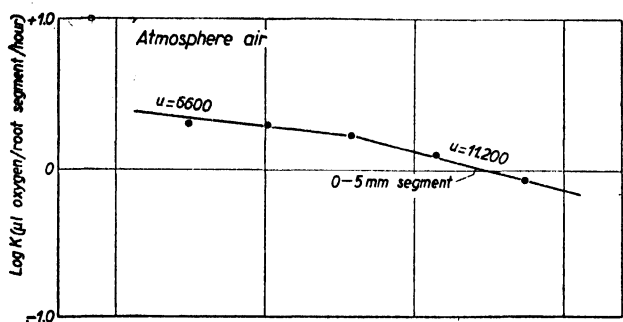


Fig. 7. Log K ( $\mu$ l of oxygen consumed /root segment/ h) plotted against the reciprocal of the absolute temperature for different root zones in an atmosphere of 5% oxygen

process, the critical increment should be about 4500 calories at 25° C. 4300 calories is in particularly close agreement with the value given by MOELWYN-HUGHES. The



Figs 8, 9, and 10.  $\log K$  (μl of oxygen consumed /root segment/ h) plotted against the reciprocal of the absolute temperature for the 0-5 mm zone of the root in an atmosphere of 10% oxygen, 15% oxygen, and 21% oxygen (air)

seen for the two basal curves at this oxygen pressure compared with those at 100% oxygen. This suggestion is in line with the criterion set up by BURTON<sup>26</sup> for the relative velocity of the "master" reaction. It must be only 1/10 as fast as the next slowest link in a catenary system or else the observed velocity may be an average of several reactions proceeding in sequence.

Since only the 0-5 mm segment was found to undergo a major change in temperature characteristic at 5% oxygen, the results at successively higher oxygen pressures are

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question naturally arises as to the basis for the large slopes seen in the other two curves. In the first place, the points at 35° C are completely off the curves and might have been included as breaks in the curves with different slopes. By inspection, it is seen that a line joining the points at 30° C and 35° C for the 10-15 mm segment of root would be parallel with the curve for the 0-5 mm segment. The points at each of these temperatures for the 5-10 mm segment fall almost on a horizontal line (slope = 0) and may be due to the inactivation of the respiratory mechanism at the higher temperature. At temperatures below 30° C the fraction of the total respiring volume of root that might be dependent upon diffusion is probably small compared to that for the apical 5 mm. It has been calculated (see BERRY AND NORRIS<sup>25</sup>) that in 5% oxygen at 30° C more than one-half of the 5-10% mm segment is aerobic. Even though the pressure of oxygen at 5% is a limiting factor in the metabolic rate, it is limiting only a small part of the whole and its contribution might be masked by the small decrease in slope that can be



shown only for this segment of the root. At 10% oxygen the points fall on straight lines, as shown in Fig. 8, with a break occurring at 25° C. Similar curves are shown in Figs 9 and 10 for 15% and 21% (air) oxygen, respectively. In all three figures a break is seen at 25° C and the slope of the left hand portion of the curves progressively increases from 1600 calories at 10% oxygen to 2500 calories at 15% and 6600 calories in air. The right hand curves increase from about 10000 calories to 11200 calories. The last value agrees within extremely narrow limits with those found for the two more basal regions of root at 5% oxygen (Fig. 7).

#### DISCUSSION

The hyperbolic curves relating pressure of oxygen to rate of respiration for each segment of root at different temperatures are consistent with most of the experimental data found in the literature. The pressure of oxygen, below which oxidative metabolism becomes diminished, is generally dependent upon three factors: (1) the size of the material under investigation; (2) the velocity of oxygen consumption per unit of tissue; and (3) the permeability of the tissue to oxygen. Both (1) and (2) are in direct proportion to the critical pressure while (3) is inverse. In the present experiments the size remains constant under all conditions and (2) varies with temperature. Figs 1, 2, 3, and 4 show, by comparison, an increasing critical pressure as the respiratory rate is elevated. The importance of permeability is considered in some detail by BERRY AND NORRIS<sup>25</sup>, but there is evidence to support the assertion that considerable differences may be expected between cells and tissues of various origin.

It is particularly interesting to note that at temperatures of 25° C (see BERRY<sup>13</sup>) or above, the apical 5 mm of root are partially anaerobic in air and that this condition extends up into the 5-10 mm zone at temperatures above 30° C. From an ecological point of view, the question naturally arises as to the significance of this fact and its bearing on the economy of the plant. Do the optimum conditions for growth depend at least in part upon this relationship? It could possibly account for the long recognized dependency of root growth on oxygen tension, and the standard procedure of growing onion roots in an aerated aquarium. So far as the authors are aware no data are available for the relative growth rates of onion roots at different oxygen tensions, but there is the general impression (frequently of doubtful significance) that more rapid growth occurs when the medium is aerated. It was observed, moreover, throughout the duration of the present experiments that when the growth temperature of the onion roots fell below 25° C, thicker roots were obtained. Since the onion root has been used extensively in working out the relationship between inherent electrical potentials and cellular oxidation (for bibliography see ROSENE<sup>28</sup>), the most favourable temperatures may not always have been employed for this purpose. In fact, BERRY AND HOYT<sup>29</sup> found that the magnitude of externally applied current, which caused the apex of the root to become electro-negative to a basal region of the root as measured in an external circuit (instead of its usual positivity), was reduced at lower temperatures and that at about 30° C no such effect could be produced. Whether this is dependent upon an anaerobic core, upon the velocity of oxidation, or some other factor(s) remains unanswered.

Two facts point to diffusion as the primary limiting factor in rate of oxygen consumption by the root segments at oxygen tensions below the critical pressure. First, the respiratory quotients consistently exceed unity under these conditions, indicating

that anaerobic carbon dioxide is being produced. Second, the temperature characteristics of those curves in which the rate of respiration was most significantly lowered by a reduced oxygen pressure are in general agreement with values for diffusion<sup>19, 23, 24</sup>. It is obvious that we are dealing with more than a simple physical system in which diffusion alone is involved. An attempt to visualize the condition in the 0-5 mm segment at 15° C in 5% oxygen will illustrate the complexity. Oxygen diffuses into the cylinder of cells and is consumed as it passes toward the axis. Therefore an outer sleeve of aerobic cells extending into the root a distance X from the surface surrounds a cylindrical core of anaerobic cells in the center. The rate of respiration as measured manometrically depends upon the size of X (*i.e.*, the number of aerobic cells), the rate of diffusion (how fast molecular oxygen reaches the loci of combination), and, possibly, on the mass action relationship between concentration of oxygen and concentration of enzyme with which it combines. This last suggestion implies an interaction between molecular oxygen, as substrate, and an aerobic oxidase (such as cytochrome oxidase) comparable to that known to hold for the majority of enzymatic changes<sup>30</sup>. However, it is generally assumed (*cf.* 20) that the rate of oxidation of the enzymes directly linked with molecular oxygen is independent of the concentration of oxygen even though an experimental proof is lacking. In those enzyme reactions where mass action does apply, essentially the same type of hyperbolic curves as those seen in Figs 1, 2, 3, and 4 as well as in the curve for the 0-5 mm segment in Fig. 5 would result. In each case a Michaelis "constant"<sup>30</sup> could be calculated, but it would not be obtained under conditions that would permit such a calculation and would be without meaning.

When the temperature is raised around the apical segment in 5% oxygen, an increased respiratory rate is obtained but the proportionate increase is less than it is in 100% oxygen. If the enzyme concentration is constant, and if the rate of oxygen consumption is independent of oxygen concentration, the only explanation for the experimental observation is to assume that X is smaller. The limiting factor in determining the magnitude of change in velocity of respiration would seem to be, therefore, rate of diffusion. No proof can be offered for the assumptions made in reaching this conclusion. Nevertheless, a constant rate of oxygen consumption with time at temperatures below 35° C was found for all root zones when the oxygen tension exceeded the critical pressure. This suggests that no progressive change in enzyme concentration occurred. Since this was not true at 35° C, this temperature was not used in any calculations shown in the  $\log k$  versus  $1/T$  curves in Figs 6, 7, 8, 9, and 10.

The changes in the  $\mu$  values seen in Figs 8, 9, and 10 require some comment. The lower values to the left of the break in the curves become progressively larger as the pressure of oxygen increases (1600, 2500, 6600 calories per mole which should be compared with 4300 calories per mole in Fig. 7). Similarly the values to the right of the break change from 10300 to 9900 to 11200 calories per mole and these should be compared with the 13800 calories per mole in Fig. 6. There are three ways to interpret these changes: (1) the reaction giving rise to the  $\mu$  value is different at each partial pressure of oxygen and possesses a different temperature characteristic; (2) the range of the different  $\mu$  values represents the error in calculation; and (3) the probability of activation becomes progressively larger as the pressure of oxygen increases due to increased randomness and hence the entropy term, which appears in the equation relating the velocity constant to both the change of heat content and the change in entropy of the activated complex, becomes larger, thereby accounting for the changing  $\mu$  values. The

second interpretation is certainly the most obvious and probably the most likely even though the third cannot be entirely dismissed without consideration.

A temperature characteristic around 12000 calories per mole for respiration of the onion root is smaller by about 4000 than that reported by other workers for respiration of various biological materials of animal origin (see CROZIER<sup>31</sup>). The significance of this difference is obscure and it is doubtful whether it is worthy of much discussion. The proposal of CROZIER that this should represent the mastery of a different reaction when  $\mu$  values varying by this amount appear has been vigorously attacked by BOOIJ AND WOLVEKAMP<sup>22</sup>. Their principal objection may be given in their own words as follows: "The main conclusion must be that as far as steady state processes are concerned the conception for the master process in physical chemistry as well as in biology is only of a limited value and that master reactions in the strict sense of the word do not occur at all . . . If the complexity of processes like that of metabolic processes is great a mathematical treatment of the whole is very nearly useless." However, they admit that a biochemical process may be limited in borderline cases by such things as oxygen tension in the habitat of the biological system. MORALES<sup>27</sup> has recently answered these objections by showing the conditions under which a steady state enzymatic system of consecutive reactions permits kinetic analysis with some accuracy. This is permissible when it is assumed that the velocity of the irreversible splitting of the enzyme-substrate complex is slow and is dependent on the product of the velocity constant and total enzyme concentration, *i.e.*:

$$V = k [E]$$

He believes that the JOHNSON-EYRING<sup>32</sup> scheme developed for the luciferin-luciferase reaction fulfils these requirements. It should be noticed that similar assumptions were made above in considering the validity of the  $\mu$  value for diffusion. The ultimate significance of these calculations therefore depends upon a proof of the accuracy of the assumptions.

#### SUMMARY

The rate of oxygen consumption and carbon dioxide production in different partial pressures of oxygen at 15°, 20°, 30° and 35° C were measured manometrically in segments of onion root tip 5, 10 and 15 mm in length. The rates for the regions 5-10 mm and 10-15 mm above the tip were determined by difference. Curves for the three zones of root tip relating oxygen uptake to pressure of oxygen were hyperbolic at all temperatures but the pressure just supporting the maximum rate (critical pressure) increased with temperature. R.Q.'s were essentially unity at pressures equal to or greater than the critical pressure and exceeded unity when the pressures were less than the critical pressure. Rate of carbon dioxide production paralleled that of oxygen consumption but showed a smaller proportionate reduction at low oxygen tensions. Log  $k$  vs  $1/T$  curves for each root segment at different pressures of oxygen permitted calculations of activation energy. For the two basal zones,  $\mu$  values of approximately 12000 calories were obtained at all pressures. For the apical 5 mm, a  $\mu$  value of 4300 calories was found at 5% oxygen and one of about 13000 calories at 100% oxygen. The curves at pressures of 10%, 15%, and 21% oxygen for the same segment had breaks with the slopes of the two straight-line portions approaching these extremes. From these results, it is concluded that diffusion is the limiting factor in rate of respiration at the lower pressures of oxygen.

#### RÉSUMÉ

Nous avons mesuré avec un manomètre, sous diverses pressions partielles d'oxygène à des températures de 15°, 20°, 30° et 35° C, le taux de la consommation de l'oxygène et celui de la production du CO<sub>2</sub> dans des segments de l'extrémité d'une racine d'oignon ayant 5, 10, et 15 mm de longueur. Les taux pour les zones situées entre 5 et 10 mm et entre 10 et 15 mm au-dessus de l'extrémité ont

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été déterminés par différence. Les courbes qui montrent les rapports entre la consommation et la pression de l'oxygène dans les trois zones de la racine étaient hyperboliques à toutes les températures; mais la pression qui maintenait tout juste le taux maximum (la pression critique) augmentait avec la température. Les quotients de respiration (R. Q.'s) étaient essentiellement l'unité à des pressions qui égalaient ou qui dépassaient la pression critique, et ils excédaient l'unité quand les pressions étaient plus basses que la pression critique. Le taux de la génération du  $\text{CO}_2$  était parallèle à celui de l'oxygène, mais il montrait une moindre diminution proportionnelle à de basses tensions d'oxygène. Les courbes  $\log k$  vs  $1/T$  pour chaque segment de racine à des pressions différentes d'oxygène ont permis les calculs de l'énergie d'activation. Pour les deux zones de base, nous avons obtenu des valeurs  $\mu$  d'à peu près 12000 calories à toutes les températures. Pour les 5 millimètres apicaux, nous avons trouvé une valeur  $\mu$  de 4300 calories à 5 % d'oxygène, et une valeur  $\mu$  d'à peu près 13000 calories à 100 % d'oxygène. Les courbes pour le même segment aux pressions de 10, 15 et 21 % d'oxygène n'étaient pas continues; l'inclinaison des deux segments rectilignes s'approchait de ces extrêmes. De ces résultats nous avons conclu que la diffusion est le facteur limitatif du taux de respiration aux pressions inférieures de l'oxygène.

### ZUSAMMENFASSUNG

Die Geschwindigkeit der Sauerstoffaufnahme und der Kohlendioxydabgabe bei verschiedenen Partialdrucken von Sauerstoff bei 15, 20, 30 und 35° C wurde manometrisch in Zwiebelwurzelsegmenten von 5, 10 und 15 mm Länge gemessen. Für die 5–10 mm und 10–15 mm oberhalb der Spitze der Wurzel gelegenen Zonen wurde die Geschwindigkeit durch Subtraktion bestimmt. Die Kurven, welche die Sauerstoffaufnahme in Abhängigkeit vom Sauerstoffdruck bringen, verlaufen für die drei Zonen der Wurzelspitze bei allen Temperaturen hyperbolisch, aber der Druck welcher gerade die grösste Geschwindigkeit aufrecht erhielt (der kritische Druck), nahm mit der Temperatur zu.

Die Atmungskoeffizienten (R.Q.) waren gleich 1 bei Drucken die grösser oder gleich dem kritischen Drucke waren. Die Abgabe von Kohlendioxyd verlief parallel zur Sauerstoffaufnahme, aber die proportionale Abnahme bei niedrigen Sauerstoffdrucken war geringer. Auf Grund der Kurven welche für jedes Wurzelsegment bei verschiedenen Sauerstoffdrucken den  $\log k$  in Abhängigkeit von  $1/T$  bringen, konnte die Aktivierungsenergie berechnet werden. Für die zwei Grundzonen wurden für  $\mu$  bei allen Drucken Werte von ungefähr 12000 Kalorien gefunden. Für die ersten 5 mm von der Spitze haben wir bei 5 % Sauerstoff  $\mu = 4300$  Kalorien und für 100 % Sauerstoff ungefähr  $\mu = 13000$  Kalorien gefunden. Die Kurven für Drucke von 10 %, 15 % und 21 % Sauerstoff für ein und dasselbe Segment bestanden aus zwei geradlinigen Stücken deren Neigung sich diesen beiden Grenzwerten näherte. Aus diesen Ergebnissen folgern wir, dass die Diffusion der Faktor ist, der die Atmungsgeschwindigkeit bei niedrigen Drucken begrenzt.

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## STUDIES OF ONION ROOT RESPIRATION

## II. THE EFFECT OF TEMPERATURE ON THE APPARENT DIFFUSION COEFFICIENT IN DIFFERENT SEGMENTS OF THE ROOT TIP\*

by

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The exchange of many materials between cells or tissues and their immediate environment is dependent upon the process of diffusion. This fact has been recognized for years and numerous investigations have been devoted to the subject. The review by JACOBS<sup>1</sup> gives a comprehensive treatment of diffusion under various conditions applicable to the living system. In spite of the volume of work on the general subject, little has been done specifically with diffusion of oxygen into higher plant tissues. Only WANNER<sup>2</sup> and BERRY<sup>3</sup> have calculated coefficients of diffusion, and in both cases the experimental material was the onion root. The values obtained differed by a factor of from 3-5, which may have been due at least in part to the techniques. WANNER used a polarographic method and BERRY the Warburg constant volume manometer.

The diffusion of oxygen into animal cells and tissues has been more thoroughly studied. KROGH<sup>4</sup> calculated coefficients of diffusion for sheets of muscle and connective tissue from data on the rate of transfer of oxygen across these materials serving as membranes. The coefficients obtained have been used extensively as characteristic of most living systems. Subsequently, additional determinations have shown a considerable range of values to exist<sup>5</sup>. This is particularly important in connection with the widespread use of manometric techniques for measuring oxygen consumption, since the rate of diffusion sets the limit of thickness of the experimental material under investigation<sup>6</sup>. Diffusion also may be capable of acting as the limiting factor in the kinetics of metabolic processes. This was strongly suggested as the case for the apical 5 mm of onion root at 5% oxygen<sup>7</sup>. Since no reference can be found to a complete analysis of diffusion in tissues of different age at several temperatures, the present investigation was undertaken as a natural sequel to the results presented in the previous papers<sup>3, 7</sup>. It is believed that for the first time evidence is given that clearly shows the limits imposed on a root system by diffusion. This is of interest especially because masterprocesses are being subject to question<sup>8</sup>.

## METHODS

In order to calculate the diffusion coefficient of oxygen for the onion root, the equation inde-

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penderly derived by FENN<sup>9</sup> and GERARD<sup>10</sup> for diffusion into a cylinder is used. The equation states that

$$U = C - \frac{a}{4D} (R^2 - r^2) \quad (1)$$

where  $U$  is the pressure of oxygen at the desired point in the tissue and  $C$  is the external pressure of oxygen, both in g per ml;  $a$  is the volume of oxygen consumed in g per ml of tissue per second;  $R$  is the radius of the cylinder in centimeters;  $r$  is the radial distance in centimeters from the cylinder axis where  $U$  is wanted; and  $D$  is the coefficient of diffusion in units of  $\text{cm}^2$  per second. When conditions are such that the pressure of oxygen ( $U$ ) is zero just at the axis of the cylinder then  $r = 0$  and the equation reduces to:

$$D = \frac{a R^2}{4 C} \quad (2)$$

It is possible, therefore, to solve for  $D$  when the pressure of oxygen in the environment ( $C$ ) is just great enough to support the maximum rate of respiration. This pressure, the critical pressure, was determined for three segments of onion root; namely, the region 0–5 mm above the tip which is essentially a zone of mitotically active cells; the region 5–10 mm above the tip which is essentially a zone of elongation; and the region 10–15 mm above the tip which is a zone of differentiation. The Warburg constant volume manometric technique was employed. The procedure followed is described by BERRY<sup>3</sup>, and the data have been given in the previous paper by BERRY AND NORRIS<sup>7</sup>. The value of  $R$  is taken from BERRY.

The necessary assumptions implied in the calculation of  $D$  and the limitations of the method are considered in an earlier paper<sup>3</sup> and will not be repeated here.

## RESULTS

The data necessary for the calculation of the coefficients of diffusion for each of the three zones of onion root at temperatures of 15°, 20°, 30° and 35° C are given in Table I along with the values of the coefficients. The volume of each root segment which was used in converting the rate of oxygen consumption as  $\mu\text{l}$  per root per hour into g per ml of root tissue per second was determined as the volume of a cylinder having a diameter of 0.073 cm and a height of 0.5 cm. In the case of the apical root zone where the tip is tapered, the volume is less than that actually used in the calculation. If it is assumed that the first millimeter of the root tip is a cone, the values of  $D$  for the 0–5 mm segment would be about 15% larger than those given in the table. This does not take into consideration the undetermined reduction in the value of  $R^2$  which would be necessary if the conical shape of the very tip is used. It appears therefore that the error is less than 15% because of this simplification.

The coefficient of diffusion for the apical zone increases with temperature from 15° to 30° but declines from 30° to 35° C. The latter is due to the decrease in " $a$ " at the higher temperature which appears to be the result of heat inactivation of enzymes (see Fig. 5 of previous paper<sup>7</sup>). If the rate of oxygen uptake per hour is calculated from the rate during the first 15 minutes of the run a value of 3.06  $\mu\text{l}/\text{root}/\text{hour}$  is obtained and when this is used in solving for  $D$ , the value shown in parenthesis in Table IA, column 6, is obtained. For both the 5–10 mm and the 10–15 mm segments, the diffusion coefficients at 30° seem to be out of line with the values for other temperatures. It would appear that the critical pressures are actually higher than the measurements indicated. The coefficients for the other temperatures have the same trend as the apical segment in showing a progressive increase with temperature.

In Fig. 1, plots are made of  $\log D$  for each root zone *versus* the reciprocal of the absolute temperature. This permits the calculation of the Arrhenius  $\mu$  (or critical increment) from the slope of the curve, the numerical values of which are shown. This

TABLE I  
DATA USED IN SOLVING FOR DIFFUSION COEFFICIENTS

A  
0-5 mm Segment

Temperature °C	a*		C*		D
	$\mu\text{l}/\text{root}$	$\text{g}/\mu\text{l}/\text{sec}$	%O <sub>2</sub>	$\text{g}/\mu\text{l}$	$\text{cm}^2/\text{sec}$
1	2	3	4	5	6
15	0.85	$1.529 \cdot 10^{-7}$	15	$0.720 \cdot 10^{-5}$	$7.077 \cdot 10^{-6}$
20	1.24	$2.192 \cdot 10^{-7}$	21	$0.910 \cdot 10^{-5}$	$8.028 \cdot 10^{-6}$
30	2.78	$4.751 \cdot 10^{-7}$	45	$1.606 \cdot 10^{-5}$	$9.843 \cdot 10^{-6}$
35	2.68	$4.509 \cdot 10^{-7}$	50	$1.648 \cdot 10^{-5}$	$9.114 \cdot 10^{-6}$ ( $10.40 \cdot 10^{-6}$ )

B  
5-10 mm Segment

15	0.40	$0.719 \cdot 10^{-7}$	10	$0.480 \cdot 10^{-5}$	$4.992 \cdot 10^{-6}$
20	0.56	$0.990 \cdot 10^{-7}$	15	$0.650 \cdot 10^{-5}$	$5.065 \cdot 10^{-6}$
30	1.45	$2.478 \cdot 10^{-7}$	21	$0.750 \cdot 10^{-5}$	$11.04 \cdot 10^{-6}$
35	1.87	$2.144 \cdot 10^{-7}$	40	$1.318 \cdot 10^{-5}$	$7.961 \cdot 10^{-6}$

C  
10-15 mm Segment

15	0.33	$0.593 \cdot 10^{-7}$	10	$0.480 \cdot 10^{-5}$	$4.12 \cdot 10^{-6}$
20	0.45	$0.795 \cdot 10^{-7}$	10	$0.434 \cdot 10^{-5}$	$6.117 \cdot 10^{-6}$
30	0.75	$1.282 \cdot 10^{-7}$	10	$0.357 \cdot 10^{-5}$	$11.04 \cdot 10^{-6}$
35	1.30	$2.186 \cdot 10^{-7}$	30	$0.988 \cdot 10^{-5}$	$7.368 \cdot 10^{-6}$

\* The values shown under columns 2 and 4 taken from BERRY AND NORRIS<sup>7</sup>.

The radius of the onion root used in calculating the volume of a given segment was 0.0365 cm taken from BERRY<sup>3</sup>.

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type of graphical representation is permissible only if  $D$  is a rate constant equivalent to the specific reaction rate constant for a chemical process\*. MOELWYN-HUGHES<sup>11</sup> states that under conditions where the rate of a heterogeneous reaction is determined by a diffusion process,  $dk/dT$  should equal  $dD/dT$  and the critical increment should thus be about 4500 calories at 25° C. Since  $D$  was obtained under conditions when diffusion appears to be just limiting, the graphical procedure seemed justified.

The curve for the 0-5 mm segment of root in Fig. 1 falls exactly on the points for all temperatures except 35° C (see above). The critical increment was calculated as 3900 calories per mole. The 4100 calories per mole for the 5-10 mm curve checks closely with the apical zone. There is more scatter of the points in this case and the 30° C value is omitted entirely. However, there is included a value for 25° C which was taken from BERRY<sup>3</sup>. The graph for the results obtained with the 10-15 mm root zone gives a straight line for all points (including one for 25° taken from the earlier work<sup>3</sup>), except

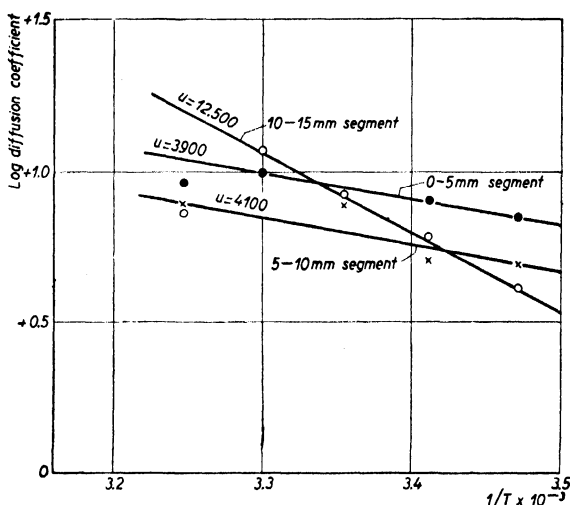


Fig. 1. Logarithm of the diffusion coefficient for different root zones plotted against the reciprocal of the absolute temperature

for 35° C. The critical increment in this case, 12500 calories per mole, is identical to the value found for rate of oxygen consumption by the same zone (see Fig. 6 of BERRY AND NORRIS<sup>7</sup>). The critical increments for the zones 0-5 mm and 5-10 mm above the tip of the root are in good agreement with the value predicted by MOELWYN-HUGHES<sup>11</sup> and with those reported for diffusion of simple solutes in water<sup>12, 13</sup>. They are also in excellent agreement with the critical increment of rate of oxygen consumption by the apical 5 mm of the root at 5% oxygen (see Fig. 7 of BERRY AND NORRIS<sup>7</sup>), when diffusion of oxygen was clearly the limiting factor. The results with the 10-15 mm segment must, as previously suggested, be in error and serve as a good example of diffusion coefficients calculated under conditions when the critical pressures for diffusion have not been precisely determined. On the evidence presented in the first paper of this series<sup>7</sup> along

\* The reaction rate constant ( $k$ ) has dimensions of number (mol, mass, ml, etc.) per unit time and the diffusion coefficient ( $D$ ), which is actually a constant for certain concentration ranges, also has dimensions of number per unit time but with the addition of per unit area. Even  $k$  has implied in it a volume term.

with that shown in the first two curves of Fig. 1, it would seem conclusive that metabolism and not diffusion was limiting in the 10–15 mm zone. It would also appear that a  $dD/dT$  plot, as MOELWYN-HUGHES<sup>11</sup> states, offers an excellent criterion for a heterogeneous reaction limited by diffusion when the critical increment is found to be approximately 4500 calories. Fig. 1 gives examples therefore of results for and against diffusion as a limiting process.

One of the most important assumptions that must be made in calculating the diffusion coefficient for a cylinder is a uniform rate of respiration from the surface to the axis. The validity of this assumption can be tested with the data for rate of oxygen consumption by the different segments at pressures of oxygen lower than the critical pressure, the pressure at which  $D$  was calculated and the lowest pressure supporting the maximum metabolic rate (see Figs 2, 3, and 4 of previous paper). If equation (1) is used to solve for  $r$ , when  $U$  is zero at a distance  $r$  from the axis, with  $C$  and “ $a$ ” known

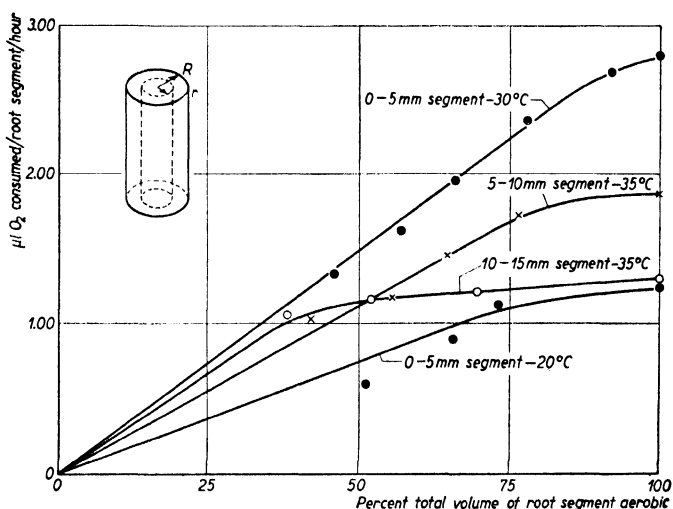


Fig. 2.  $\mu\text{l}$  of oxygen consumed/root/segment/h as a function of the percent of the total volume of the root segment that is aerobic. Curves for different root zones at different temperatures are shown.

from the experiments and  $D$  calculated above, then the volume of the anaerobic cylinder (the dotted zone in the inset in Fig. 2) can be found. When this volume is compared to the total root volume, the percent of the root that is aerobic is then known. This can be plotted against the measured rate of oxygen consumption (in  $\mu\text{l}$  of oxygen per root per hour) as shown by the four typical curves in Fig. 2. The original assumption would necessitate a linear relationship in these graphs but it can be seen that this does not hold. In every case, the curve levels off as the center of the root is approached. This was true for every root zone at every temperature in which the curves in Fig. 1 suggest that the values for  $D$  are reliable. In three of the four curves the break falls between 75% and 100% of the root aerobic and only curve 3 has the break at 50%. The value for  $D$  which was used in obtaining curve 3 is the least reliable of the four and an inspection of equation (1) shows that when  $D$  is smaller than it should be, all points will be moved to the left. Also, if  $C$  is actually smaller than the value used, an error of 1% in the pressure of oxygen would shift the points to the left four units (percent). There is reason to doubt the accuracy of curve 3 but there is no reason to believe

that a linear relationship holds in any case. If we assume the break falls between 75% and 100%, the former value corresponds to a distance where  $r = R/2$ . Histologically, this falls in the cortex and no anatomical structure is there to suggest such a change in metabolism. The stele in a segment of root 10–15 mm above the tip occupies only 3.5–4% of the volume. A much lower metabolic rate in this zone, however, might very well account for the break observed in these curves.

#### DISCUSSION

A diffusion coefficient calculated for a simple solute diffusing through a pure solvent states the quantity of material that passes unit cross-sectional area in unit time under unit concentration gradient. Since the region across which diffusion occurs in the onion root is not homogeneous, the numerical significance of the coefficient is problematical. It seems logical to presume that in reality a number of coefficients actually exist and these vary with the specific locus of the root being traversed. The single values arrived at in this report by substituting appropriate quantities in the equation that specifically applies only to a simple physical system may be considered approximate means of the many individual coefficients which it is impossible to determine. One way in which the data so obtained can be tested is to determine the activation energy as shown in Fig. 1. The fact that agreement with the theoretical value is found in two of the three cases is merely presumptive evidence for reliable results since, as MOELWYN-HUGHES<sup>11</sup> states, it is possible for reactions to possess a real energy of activation of the same value. There is no indication for this in onion root respiration, however, since in every case where diffusion seems to be completely eliminated as the rate limiting factor, the activation energy is about 12500 (as shown in Figs 6, 7, 8, 9, and 10 of the previous paper<sup>7</sup>). If we assume therefore, that diffusion coefficients having activation energies of about 4500 calories are reliable, then the values for the 0–5 mm segment and the 5–10 mm segment of onion root are acceptable and those for the 10–15 mm segment are not. Furthermore, the greater scatter of the points on the  $\log D$  vs  $1/T$  curve for the 5–10 mm segment compared to the 0–5 mm segment suggests that the coefficients for the latter zone of root are more precise than those for the former.

The above reasoning tells us nothing about the accuracy of the absolute value of the diffusion coefficient since only the rate of change in  $D$  with temperature is involved. From the curve for the 0–5 mm segment in Fig. 1 it is possible to find  $D$  for any temperature. When this is known, as well as the rate of respiration in pure oxygen at the same temperature, equation (2) can be used to calculate the critical pressure of oxygen at that temperature. If this should check with the experimentally determined critical pressure, the absolute values for  $D$ , previously found, would appear to be more reliable. In the experiments of BERRY<sup>3</sup>, air was taken as the critical pressure for the 0–5 mm segment of root at 25°C and the diffusion coefficient which was calculated was greater than the curve in Fig. 1 indicates. When the coefficient was taken from the curve, the critical pressure was found to be 30% oxygen. Subsequent experiments have verified this value. It is therefore possible to calculate the entire curve for rate of respiration *versus* pressure of oxygen from the value of  $D$  and a single experimental determination of the maximum rate of respiration; *i.e.*, the rate in pure oxygen.

Since the diffusion coefficient was found to decrease as the cells in the root become progressively older, the values reported by WANNER<sup>2</sup> come into very close agreement

with the ones shown in this report. The segment of root investigated by WANNER was basal to those used in the present study and the fact that the coefficients he found are one-half to one-third the values shown here may be due in part to the difference in age of the tissue. The coefficients of diffusion reported by KROGH<sup>4</sup> ( $6.2$  to  $7.5 \cdot 10^{-9}$ ) are practically identical to certain ones given in Table I. It is also interesting to note that when the coefficients of diffusion obtained by WANNER<sup>2</sup> are plotted as  $\log D$  vs  $1/T$ , a critical increment equal to the ones found for the apical two segments is obtained between  $15^\circ$  and  $20^\circ$  C, but between  $20^\circ$  and  $25^\circ$  C, an increment like that found for the 10–15 mm segment results. The fact that this latter value (about 12500 calories per mol) has been found repeatedly for respiratory processes in the onion root may be interpreted as strongly suggesting that a reaction, characterized by a critical increment of this magnitude, is limiting the rate of respiration.

Inasmuch as the curves of Fig. 2 indicate that the basic assumption of linear rate of respiration across the radius of the root, used in calculating the diffusion coefficient, is in error, it is important to examine the possible causes of the error and their effect on  $D$ . The neglect of the diffusion of oxygen into the ends of the root, which assumes its greatest importance when the 0–5 mm segment is used (the 5–10 mm zone and the 10–15 mm zone were studied by using 10 and 15 mm lengths of root respectively), would increase the area of diffusion by about 7%; this would give an apparent diffusion coefficient larger than the true value. This is in the opposite direction from the change in  $D$  which would result if the metabolic rate were linear across the entire radius of the root. Thus if each curve in Fig. 2 were extended as a straight line to the 100% aerobic volume,  $D$  would be increased. The sum of these effects on  $D$  are probably not equal and opposite and the most likely result would seem to be a diffusion coefficient somewhat larger than the true value. However, the small departure from linearity in three of the curves of Fig. 2 suggests that no gross error results from the assumption when the experimental measurements are precise. Therefore, when diffusion coefficients calculated in the manner described in this paper withstand these tests of reliability, it is believed that they closely approximate the true apparent rate of penetration of oxygen into the living tissue and very strongly suggest that diffusion is the limiting factor in respiratory rate under certain conditions.

#### SUMMARY

The Gerard-Fenn equation for diffusion into a cylinder was used to calculate the apparent diffusion coefficient of oxygen in each of three segments of onion root tip at each of the four temperatures described in the first paper of this series.

These coefficients were then used to determine the activation energy of diffusion ( $\log D$  vs  $1/T$  graphs) with the result that those for the two most apical zones of root agreed closely with the theoretical value. Certain coefficients were also used to calculate the distance to which oxygen penetrates the root when the external pressure of oxygen is limiting. From these values it was shown that the rate of oxygen consumption across the radius of the root is not perfectly linear (as the Gerard-Fenn equation demands) but the departure from linearity is not sufficient to completely invalidate the results.

#### RÉSUMÉ

Nous avons employé l'équation de Gerard-Fenn pour la diffusion dans un cylindre pour calculer le coefficient apparent de diffusion de l'oxygène dans chacun des trois segments de l'extrémité d'une racine d'oignon aux quatre températures décrites dans la première note de cette série.

Nous avons ensuite employé ces coefficients pour déterminer l'énergie d'activation de la diffu-

sion (v. les graphiques:  $\log D$  vs  $1/T$ ); il en a résulté que les valeurs pour les deux zones les plus apicales de la racine ont répondu assez exactement aux valeurs théoriques. Certains coefficients ont été employés aussi pour calculer à quelle profondeur l'oxygène pénètre la racine à des pressions extérieures limites d'oxygène. Ces valeurs nous ont permis de constater que le taux de consommation de l'oxygène le long du rayon de la racine n'est pas tout à fait linéaire (comme l'exige l'équation de Gerard-Fenn); mais ce défaut de linéarité ne suffit pas à infirmer complètement les résultats.

### ZUSAMMENFASSUNG

Auf Grund der Gleichung von Gerard-Fenn für die Diffusion in einem Zylinder wurde der scheinbare Diffusionskoeffizient für Sauerstoff in jedem der drei Segmente einer Zwiebelwurzelspitze bei den vier in der ersten Abhandlung dieser Reihe beschriebenen Temperaturen berechnet.

Mit Hilfe dieser Koeffizienten wurde hierauf die Aktivierungsenergie der Diffusion bestimmt (vgl. die Diagramme  $\log D$ :  $1/T$ ); für die beiden der Spitze am nächsten gelegenen Segmente stimmten die gefundenen Werte mit den theoretischen gut überein. Auf Grund einiger Koeffizienten wurde auch berechnet, wie weit der Sauerstoff in die Wurzel eindringt, wenn der äussere Sauerstoffdruck verschiedene Grenzwerte hat. Diese Werte liessen erkennen, dass der Sauerstoffverbrauch dem Wurzelradius entlang nicht ganz linear verläuft (wie es die Gleichung von Gerard-Fenn voraussetzt), aber diese Abweichung ist nicht gross genug um die Ergebnisse vollkommen zu entkräften.

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## STUDIES OF ONION ROOT RESPIRATION

## III. AMPEREOMETRIC TITRATION AS A METHOD FOR THE MEASUREMENT OF RESPIRATORY OVERSHOOT\*

by

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The respiratory rebound, which has been most clearly defined by BURTON<sup>1</sup> can be measured by means of ampereometric titration. The method developed was adapted especially for onion root segments and it has made possible kinetic study of ordinary respiration rates in addition to overshoot rates produced after an anaerobic period at any temperature of experimental interest.

In contrast to ordinary polarographic analysis, where the impressed voltage across a dropping mercury electrode and an electrode consisting of a quiescent pool of mercury is increased continually, in the ampereometric technique the applied voltage is kept constant at a magnitude where the diffusion current is linearly proportional to the concentration of the material under investigation — in the case of oxygen,  $-0.5$  V. For use in respiratory studies, the decrease in the concentration of oxygen of a solution surrounding the respiring tissue can be related directly to the oxygen consumption of the tissue; this change is recorded in units of microamperes of "diffusion" current. Use of this method for determining oxygen consumption by a living tissue was first suggested by MÜLLER<sup>2</sup> for yeast suspensions. Applications to studies of photosynthetic activity have been made by PETERING AND DANIELS<sup>3</sup> and DUTTON AND MANNING<sup>4</sup>. BAUMBERGER<sup>5</sup> also employed it for measurement of respiration in yeast, in order to relate the "redox" potential to the rate of oxygen consumption. Special application to root respiration has been made by DUBUY AND OLSON<sup>6</sup> and by WANNER<sup>7</sup> who worked with *Avena coleoptile* and with *Triticum sativum* and *Allium cepa* roots, respectively.

In designing a method that could be employed for the investigation of rebound phenomena in respiration, several factors had to be taken into consideration. First the tissue (in this study, onion root segments) must be placed in a vessel permitting ampereometric titration in such a fashion that there is no physical contact either with the dropping mercury or with the quiescent mercury pool, not only because the mercury might be toxic to the living tissue, but also because there must be no mechanical hindrance on the mercury drop as it forms, ripens and falls, if reproducibility is to be at-

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tained. Second, the vessel must be designed in such a fashion that rapid changes in liquid environment (from initial aerobiosis to anaerobiosis back to aerobiosis) are possible without loss in number of roots, without physical damage to the tissue, and with minimum liquid turbulence. Third, the vessel has to be constructed in such a fashion that the volume occupied by the tissue and the solution is a constant one. This means that there must be some device for the removal of excess mercury as it accumulates. Fourth, and finally, it must be possible to place the entire system in a water bath in order that it be kept at constant temperature.

The cell and inset designed to meet these requirements is shown in Figs 1 and 2. The cell is a modification of that used first by DuBUY AND OLSEN<sup>6</sup> and later by WANNER<sup>7</sup>. Fig. 2 shows an isometric representation of the Plexi-Glass\* support employed inside the cell. The inset was machined in such a fashion that the top disc could be easily removed to permit admission of the root segments but would not come off as the liquid

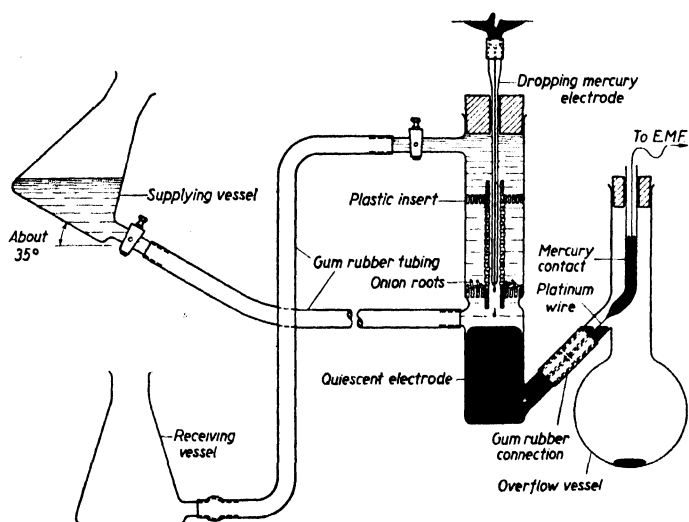


Fig. 1. Cell with inset for the polarographic determination of oxygen consumption before and after an anaerobic period

was changed. As many holes as possible of the size shown in Fig. 2 (about 0.75 mm) were drilled in both shoulders and in the center cylinder to permit diffusion to the drops issuing from the capillary, shown in operating position in Fig. 1. The DuBUY-OLSON cell was further modified by the introduction of two side-arms, one placed with its lowest edge flush with the top of the mercury meniscus of the quiescent electrode, the other, above the top of the uppermost shoulder of the plastic inset. To the top side arm there was attached a micro stop-cock. The side arms were subsequently connected by means of gum rubber tubing to two Erlenmeyer flasks of 50 ml capacity. At the bottom of each of these there was affixed a piece of glass tubing in order that connection might be made with the electrolysis cell; in addition, the flask to be attached to the lower of the cell side arms was also equipped with a micro stop-cock. The tubing length between the receiving vessel and the electrolysis cell was 18.5 cm, that between the supplying vessel and the electrolysis cell was 30.0 cm. Before use the tubing was extracted con-

\* Plexi-Glass supplied by Dr C. E. ANDERSON of the Rohm and Haas Co., Philadelphia, Penna.

tinuously for 18 hours with boiling acetone in order that any free sulphur might be removed. The entire cell, with the exception of the two Erlenmeyer flasks was placed in a Plexi-Glass clamp (not shown in the figure) so constructed that the vessel containing the roots and the vessel for mercury overflow could be kept at a fixed distance from one another. The clamp fastened by means of a brass set screw to a brass rod of such length that it could be placed in the water bath. The end of this rod rested on a rubber pad which served as a damp for vibration in the bottom of the bath.

The receiving Erlenmeyer flask was placed in the water bath, the supplying flask above it, approximately in the position indicated in Fig. 1. These positions, particularly the angle of the supplying vessel, and the length of the connective tubing were important in keeping turbulence at a minimum while changing the solutions. Adherence to the specifications given above permitted flushing through of 30 ml of solutions (about 7 ml more than the actual volume of the electrolysis cell) in 23 seconds. The effect of this manipulation on the characteristics of the respiratory record will be discussed later.

The dropping mercury electrode and the device for controlling the amount of pressure on the drop were constructed according to KOLTHOFF AND LINGANE<sup>8</sup>. In addition the mercury reservoir and the capillary were supported on a ring affixed to a stativ so that centering and raising or lowering of the capillary was possible to within very close dimensions.

The reproducibility obtainable from the dropping mercury electrode depends to a large extent on the purity of the mercury and solutions and the cleanliness of the apparatus employed. In all of the analyses the mercury used had been previously distilled four times to insure chemical purity. Previous to distillation it was aerated in dilute nitric acid until examination showed that the surface tension of the mercury was such that contaminants had been oxidized. Following the aeration the mercury was filtered and dried with alcohol and ether and then placed in the distillation apparatus.

All platinum contacts and all glass apparatus were cleaned in the following solutions: concentrated nitric acid, dilute nitric acid, tap water, distilled water and the specially distilled water whose preparation will be described below. The plastic inset shown in Fig. 2 was aged in distilled water which contained a non-ionic wetting agent. This precaution was felt to be necessary in view of the fact that it has been found that plastics frequently discharge "Plastic-ions" into a solution if they have not been suitably aged\*. Rubber stoppers were boiled in distilled water to remove any possible contaminants.

\* Personal communication from L. G. VAN DE BOGART.

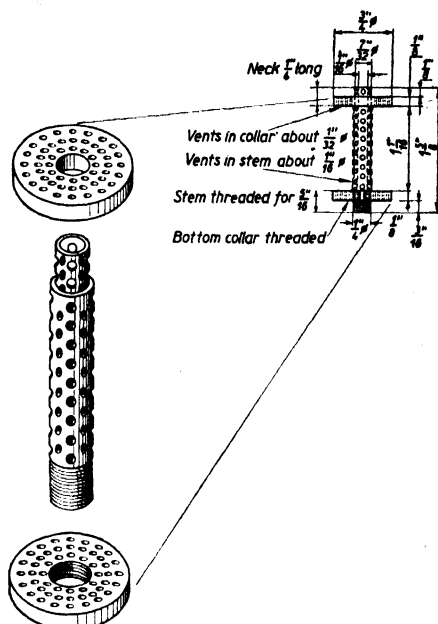


Fig. 2. Isomeric representation of Plexi-Glass inset



In order that the tank nitrogen might be oxygen free it was bubbled through seven gas-washing tubes packed with glass beads and containing the following solutions: a) a 50% (vol) solution of ammonium hydroxide and distilled water saturated with ammonium chloride, to which there had been added pieces of bright copper foil, (b, c, d) a 10% sulphuric acid solution, (e, f, g) distilled water containing a drop of methyl red. Gassing of the solutions was always carried out at the temperature of the experiment.

The specially distilled water was prepared as follows: distilled water containing sulphuric acid and potassium permanganate was redistilled and condensed in block tin. Previous to distillation it was kept at 80–90° C while purified nitrogen gas was bubbled through it. The distillation *per se* was likewise carried out under nitrogen; the condensate was stored under nitrogen.

The supporting electrolyte was a phosphate buffer of the desired  $p_H$  containing a drop of 0.1% methyl red solution for the suppression of maxima. It was found that the methyl red was best added after the solutions had been gassed, just prior to being placed in the electrolysis cell. The buffer mixtures were made of  $M/15$   $Na_2HPO_4$  and  $KH_2P_4$ .

Onion bulbs from the Barteldes Seed Company, Lawrence, Kansas, were sprouted in constantly aerated 25% HOAGLAND's solution<sup>9</sup>. The bulbs were placed on holes drilled through plastic racks so designed that the basal section of the bulb was just bathed by the nutrient, with a free circulation of the aerated solution around the bulb. The aquaria were kept in a dark room at an essentially constant temperature ( $25^\circ C \pm 2^\circ$ ). It was necessary to operate under these conditions to insure a constant growth environment and the absence of any possible growth anomalies<sup>10</sup>. Cutting of the roots was carried out under ruby light using the technique that has been described in detail by BERRY AND BROCK<sup>11</sup>. All experiments were made with 50 2 day old root segments, 5 mm in length, cut either 5 mm above the tip or at 10 and 15 mm above the tip. This meant that one of the segments had two cuts. BERRY has shown that respiration is not measurably influenced by this factor<sup>12</sup>. After cutting, the roots were placed by means of a spatula in the electrolysis cell containing about 17 ml of the oxygenated buffer solution. This was made possible by removing the upper flange of the plastic support shown in Fig. 2. After the roots had been added, the flange replaced, and the cell stoppered, the capillary was slowly lowered into such a position that the tip of the capillary was in approximately the same plane as the upper surface of the bottom flange, as the diagram shows. The cell was then completely filled with oxygenated buffer by means of the supplying vessel. Care was taken to obtain a continuum of fluid from the stop cock on the supplying vessel to the stop cock on the upper arm of the cell. This was insured by employing an excess volume of buffer.

At each temperature of measurement it was necessary to make two calibrations: the variation in drop time with applied pressure, and the variation in limiting current with oxygen concentration. The first of these was accomplished by the use of the apparatus described by LINGANE AND KOLTHOFF<sup>13</sup> for the collection of mercury drops at a constantly applied voltage. Because ILKOVIC<sup>14</sup>, followed by MACGILLAVRY AND RIDEAL<sup>15</sup> have shown that the diffusion current is a linear function of the quantity  $m^{1/2} t^{1/2}$ , where  $m$  is drop mass and  $t$  is drop time, this quantity was calculated from the data obtained. The plot of these values against of the values applied pressure is given in Fig. 3.

Calibration of applied current variation with respect to oxygen tension was carried

out by means of a modified Winkler technique. A sample of oxygenated buffer that did not contain any tissue was polarized in the electrolysis cell in the presence of the plastic inset. A portion of the solution was then removed and analysed according to a method recommended by the AMERICAN PUBLIC HEALTH ASSOCIATION<sup>16</sup> with the exception that the amount of iodine was estimated colorimetrically in a Coleman spectrophotometer at a wave length of 580 Å.

The Leeds and Northrup polarograph employed in these measurements was equipped with a galvanometer of the ballistic current detector type providing under the operating conditions described above a sensitivity equivalent to an oxygen concentration of  $10^{-6}$  mol/l. This factor was of great importance at the time of the switch from aerobiosis to anaerobiosis and particularly in the switch from anaerobiosis to aerobiosis, for it was found that the electrical circuit experienced its own rebound, not as a result of the change in oxygen concentration, but as a result of the fact that diffusion relationships to the forming mercury drops were upset. For this reason, special measures were taken, first, to reduce the electrical rebound to a minimum for reasons of sensitivity, and second, to guarantee that the instrumental overshoot would be limited to a known period from measurement to measurement. Satisfaction of these specifications requires a fixed position of the supplying and receiving vessels, a constant length of rubber tubing from the vessels to the cell and the use of small stop cocks rather than pinch clamps for flow control. With maintenance of the mechanical conditions previously described it was found that the root environment could be changed completely within 23 seconds. The duration of the instrumental rebound under these conditions was such that when 150 seconds had elapsed, including the 23 seconds required for emptying and refilling the vessel, the recording and amplifying circuits were in all cases again indicating the oxygen concentration of the solutions. This is shown for a change in oxygen tension in both directions at 25° C in Fig. 4. This curve is typical of numerous controls run at all temperatures at which rebound measurements were made.

In the course of the early experimental work it was soon noted that the use of the plastic inset reduced the recorded diffusion current to a value lower than that which would have been obtained in its absence. This is probably due to the fact that the support provides mechanical hindrance to diffusion to the surface of the drop. For this reason a group of experiments were made in which the center rib and the top flange of the inset were removed. Loss of roots was prevented by the introduction of a small platinum screen at the exit stop-cock; a small rubber cone whose largest diameter just slightly exceeded that of the opening of the lower flange was placed about 1 cm above.

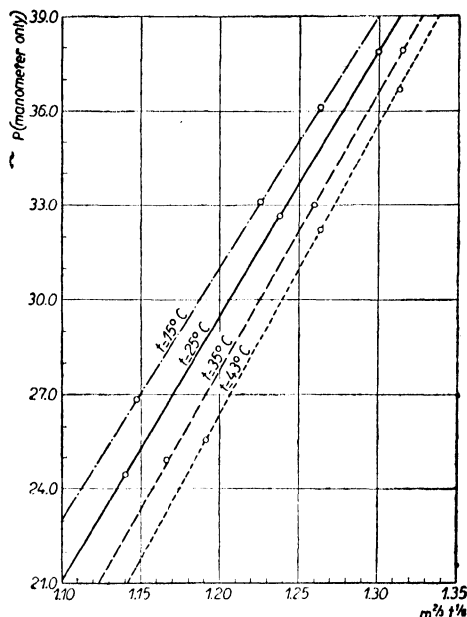
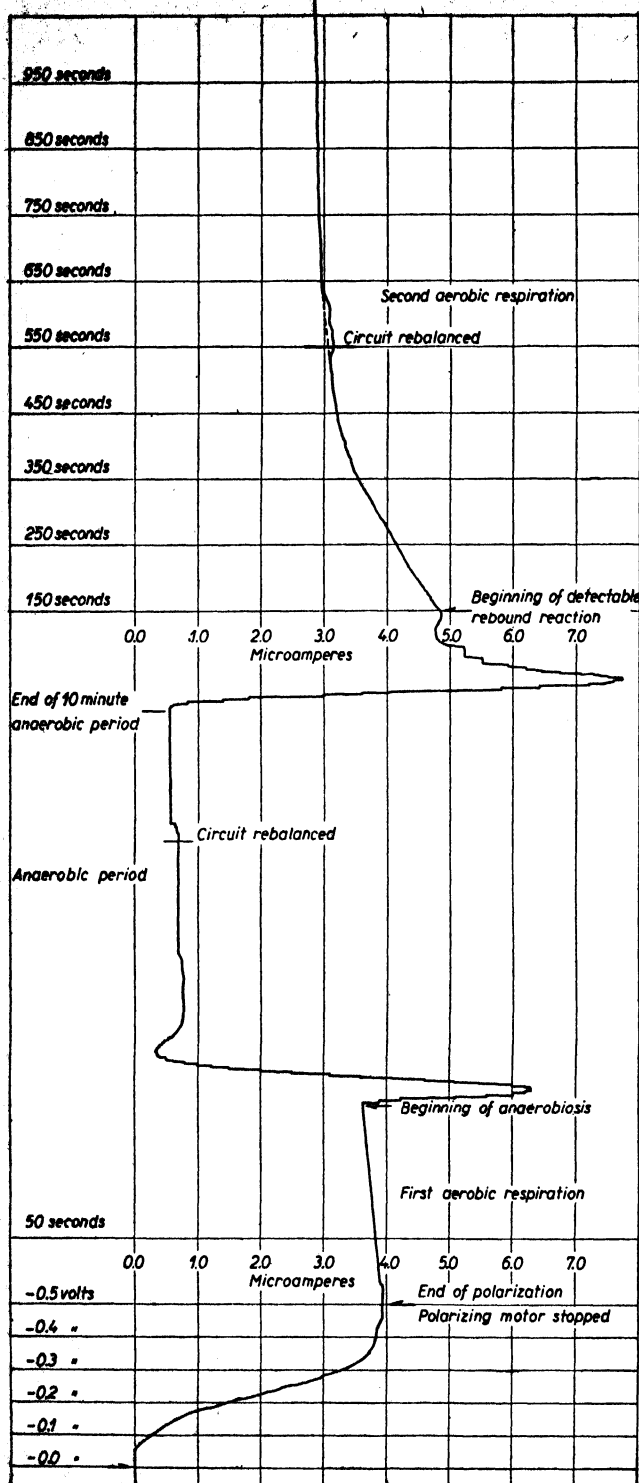


Fig. 3. Variation of  $m^2/t$  with applied pressure in centimeters.  $m$  = mass of drop,  $t$  = drop time



the tip of the capillary. At the time of the first change in solution the capillary was lowered by means of the stativ to which it was fixed until the lower opening was sealed. It was raised again after 50 seconds of the second aerobic period (the overshoot period) had elapsed. Under these conditions the instrumental rebound persisted for about 450 seconds. In addition the recorded diffusion current appeared to fluctuate sinusoidally for an additional 150 seconds. For these reasons use of this method was abandoned and the complete inset was employed.

In making an actual measurement use was made of the following procedure: the phosphate buffers were gassed at the correct temperature and the tissue placed in the cell according to the method previously described. After dropping was started, polarization was begun. The solution was polarized from 0 applied volts to  $-0.5$  V. When this point was reached the polarization was stopped and a constant voltage of  $-0.5$  V was applied. The respiration was permitted to proceed normally for 500–800 seconds and then the solution around the roots was changed to one free of oxygen. In making this

Fig. 4. Typical "ampereogram" showing electrical rebound and respiratory rebound. 50 onion root segments, 0–5 mm portion of root.  $t = 25^{\circ}\text{C}$ . 10 minute anaerobic period. pH of buffer = 6.489

change the nitrogen saturated solution was placed in the supplying vessel maintained in the position shown in Fig. 1. The stop cock connected to it was first opened and immediately thereafter, the stop cock connected to the cell was opened. When the supplying vessel was nearly drained, the latter was closed, and then the stop cock connected to the supplying vessel was closed. It was found that the sequence of these operations was of great importance in order that a minimum electrical instrumental rebound would be obtained. This is probably due to the fact changes in hydrostatic pressure above the surface of the quiescent mercury pool produced changes in its level. The roots were kept anaerobic for the desired length of time. The completeness of anaerobiosis was easily checked by observing the current delivered. In the absence of oxygen the current should exactly equal the residual current for the phosphate buffer at an applied voltage of  $-0.5$  V. When this was not the case the run was discarded. As previously stated the polarograph, under these conditions, could detect a concentration of oxygen equal to approximately  $0.5 \mu\text{l}$  in the 23 ml of buffer. At  $25^\circ\text{C}$ , 50 root segments 5 mm long at air saturation consume  $1.5 \mu\text{l}$  of oxygen per minute<sup>12</sup>. On this basis it would seem that nearly complete anaerobiosis was achieved. The change back to an environment of high oxygen tension was performed in an identical fashion. The analysis was continued until the rate of oxygen consumption returned to the initial level. No evidence of mercury poisoning was ever obtained during the time of the individual experiments since the post rebound rate of oxygen consumption was never less than that obtained in the initial aerobic period. Results of this type of measurement and an analysis of the data so obtained for different root segments, for varying periods of anaerobiosis, at different temperatures and at various concentration of hydrogen ion of phosphate buffer will be reported in subsequent papers.

Advantages of this type of rebound measurement may be most clearly seen by a comparison with other methods of measurement. A review of overshoot phenomena in nerves and muscles may be found in BURTON<sup>1</sup>; the general problem of recovery from anaerobiosis in invertebrates has been discussed by VON BRAND<sup>17</sup>; NORRIS<sup>18</sup> has carefully reviewed the existing literature on respiratory rebound and its possible relationship to rebound in electrical potential measurements.

Most of the reports in the literature have several features in common. In nearly all of the experimental work, the rebound has been treated as a static, rather than a dynamic phenomenon of the living organism. This arises, in part, from the fact that the techniques of measurement yield data which are not precisely amenable to a kinetic, dynamic treatment. Methods that have been employed include the WINKLER titration<sup>19, 20</sup>; an evaluation of the respiratory quotient, which is, in the final analysis, a static quantity<sup>21, 22</sup>, at set times in the post-anaerobic period; in the case of plant tissue, use has been made of the FENN microrespirometer<sup>23</sup>, and of the PETTENKOFER titration for carbon dioxide<sup>24</sup> and of the speed of reduction of methylene blue<sup>18</sup>. No attempt was made in any of these cases to describe the system kinetically, although in many instances, "rates" formed the basis of the discussion.

Secondly, reports are frequently characterized by lack of temperature control of the system being measured. This is perhaps the most serious indictment of a study that concerns "rates" and "rate measurements". It has been pointed out by PEASE<sup>25</sup> that if a reaction rate is doubled by a ten-degree rise in temperature (roughly true for most known chemical reactions) there may be approximately a 7% change in the reaction rate per degree, or a change of 7 parts in a 1000 for a one-tenth degree change. This

omission is even more serious when one considers the fact that theoretical considerations demand a temperature-caused rebound. However, it must be remembered that in many cases control of temperature presents serious difficulties in the designing of apparatus.

Another feature characteristic of many measurements likewise stems in most cases from the type of measurement employed for the rebound reaction. Frequently the experimental conditions are such that data are lost in the post-anaerobic period for as long a time as 5<sup>23</sup> to 90 minutes<sup>24</sup>. This should correspond to the portion of the reaction where numerical results should be of greatest interest. NORRIS's data (*loc. cit.*) have the advantage of permitting evaluation of rate from the inception of the second aerobic period.

Finally, there are examples reported in the literature for which there are no quantitative data whatsoever on the increased rate of oxygen consumption after anaerobiosis, caused, usually, by the experimental difficulties involved. Such a case is that reported by KITCHING<sup>26</sup> on the post- anaerobic response of the protozoan, *Colpatria kellicottiana*.

Use of the polarographic method permits the attainment of data 150 seconds after the inception of aerobiosis; it is capable of being carried out under controlled temperatures; metabolic waste products accumulated in the surrounding milieu during anaerobiosis are removed in the return to aerobiosis; checking of current delivered during anaerobiosis provides a means of ascertaining the completeness of the anaerobiosis, and the continuously recorded curves are amenable to kinetic analysis.

The principal limitations of the present method seem to be a) the interference with free diffusion of oxygen in the electrolysis cell imposed by the plastic inset. This apparently has little influence upon reproducibility (as shown by Tables I and II) but makes impossible the calculation of oxygen concentration in true molar units; b) the application of the technique to unicellular organisms, which offer certain advantages for kinetic studies, is impossible without modifications that pose serious technical difficulties (c) no evaluation of carbon dioxide was made along with the determination of oxygen consumption. This would provide valuable additional information.

TABLE I  
REPRODUCIBILITY OF OVERSHOOT MEASUREMENT  
0-5 mm ROOT SEGMENT  
20 Minute Anaerobic Period;  $t = 25^{\circ}\text{C}$

Time (Seconds)	Test 1 % Increase *	Test 2 % Increase *	Test 3 % Increase *
150	1082	1069	1097
200	835	800	806
250	623	596	600
300	588	590	578
350	370	367	575
400	323	296	300
450	305	280	291
500	252	267	256
550	217	212	200
600	164	171	173
650	141	133	153
700	94	83	76
750	85	52	33

\* % Increase of oxygen consumption after the anaerobic period

TABLE II  
REPRODUCIBILITY OF OVERSHOOT MEASUREMENT  
10-15 mm ROOT SEGMENT  
20 Minute Anaerobic Period;  $t = 25^{\circ}\text{C}$

Time (Seconds)	Test 1 % Increase *	Test 2 % Increase *	Test 3 % Increase *
150	465	442	402
200	408	395	340
250	377	367	389
300	229	259	238
350	200	217	189
400	170	163	159
450	164	152	157
500	108	108	100
550	92	65	87
600	--	44	13

\* % Increase in oxygen consumption after the anaerobic period

#### ACKNOWLEDGEMENT

It is with pleasure that acknowledgement is made to Dr J. L. CRENSHAW for his helpful suggestions and criticisms.

#### SUMMARY

The method of amperometric titration for oxygen, using the dropping mercury electrode, has been adapted for measuring the respiratory overshoot in segments of onion roots. The change in concentration of oxygen with time is followed continuously in the solution around the roots when an aerobic medium replaces one that is anaerobic. The period of electrical instability is limited to 150 sec, including the time required for changing the solutions. Data so obtained are amenable to kinetic treatment and reproducibility from experiment to experiment is good. The advantages and limitations of this method over those previously used are discussed.

#### RÉSUMÉ

La méthode du titrage ampérométrique pour l'oxygène, en utilisant l'électrode à goutte de mercure, est adaptée pour la mesure de l'excès respiratoire dans des segments de racines d'oignon. Les changements de la concentration de l'oxygène en fonction du temps ont été observés continuellement dans la solution entourant les racines, quand un milieu anaérobie est remplacé par un milieu aérobie. La période de l'instabilité électrique est limitée 150 secondes, le temps nécessaire pour le changement des solutions compris. Les données ainsi obtenues sont soumises au traitement cinétique, et sont reproductibles d'une expérience à l'autre. Les avantages et les limites de cette méthode, comparées à ceux des méthodes précédentes, sont discutés.

#### ZUSAMMENFASSUNG

Die amperometrische Titrationsmethode von Sauerstoff mit Hilfe der Quecksilbertropfenelektrode wurde für die Messung des Atmungsüberschusses in Segmenten von Zwiebelwurzeln angepasst. Die Änderungen der Sauerstoffkonzentration mit der Zeit wurden fortlaufend in der die Wurzeln umgebenden Lösung verfolgt, wenn ein aerobes durch ein anaerobes Milieu ersetzt wird. Die Dauer der elektrischen Schwankungen ist auf 150 Sekunden beschränkt, wobei die zum Wechsel der Lösungen benötigte Zeit mit eingerechnet ist.

Die so erhaltenen Resultate können kinetisch ausgedrückt werden und sind gut reproduzierbar. Die Vorteile und die Grenzen dieser Methode im Vergleich zu früher gebräuchlichen Arbeitsweisen werden erörtert.

*References p. 624.*

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# ON THE INTERRELATION OF RESPIRATION AND PHOTOSYNTHESIS IN GREEN PLANTS

by

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## INTRODUCTION

During a previous investigation we found anomalies in the light intensity assimilation curves measured in *Chlorella* suspensions. The linear relation between the rate of photosynthesis and the quantity of light absorbed by the cells did not hold for low intensities.

From the phenomenon, that per molecule of  $O_2$  evolved the same number of light quanta was not always used, we concluded that more than one photochemically induced process occurs in the green plant. Fig. 1 shows a typical experiment in which the rate of dark respiration (R) and a number of assimilation rates (P) at medium light intensities have been measured. The perfectly straight line  $P_1 - P_4$  cuts the ordinate at H between O and R. From these experiments we concluded that the ratio  $OH = HR$  amounted to exactly 1:1.

We interpreted this observation in the simplest way, viz., by accepting the occurrence of two photochemically induced processes. The first one ( $P_1 - P_4$ ), being normal photosynthesis, is characterized by a quantum number  $q$  (quanta absorbed per mol  $O_2$  evolved), whereas the quantum number of the second process amounts to  $\frac{1}{2} q$ .

This interpretation led to some theoretical considerations regarding both processes and we felt obliged to collect more experimental material to gain more insight into the following questions:

1. Averaging about 50 experiments we found  $OH = HR$  (Fig. 1). It seemed useful to inquire how accurately this relation holds in separate experiments. To this purpose it was necessary to use an apparatus with which measurements could be performed with a still greater accuracy.

2. For the greater part our algal material was cultivated via a medium containing glucose. It was interesting to examine whether the same phenomenon is shown by inorganically grown algae as well as by other green plants.

*References p. 631.*

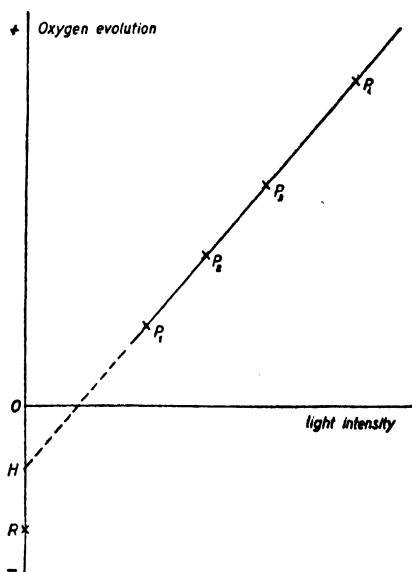


Fig. 1. Relation between light intensity and oxygen evolution according to earlier experiments



3. The assumption of only one extra photochemically induced process beside normal photosynthesis was the simplest interpretation but not the only possible one. Therefore we wished to obtain more information concerning the exact shape of the curve in the range  $R-P_1$  (Fig. 1).

#### TECHNIQUE OF MEASUREMENT

A special type of differential volumeter (KOK AND VELTKAMP<sup>6</sup>) was constructed. 10–20  $\mu$ l Algae were suspended per ml Warburg buffer no. 9.

The vessels used were filled in such a way that per  $\text{cm}^2$  irradiated bottom surface 3–6  $\mu$ l cells were present.

In this way part of the incident light was absorbed (35–50%).

The light source used was a Philips HP 300 high pressure mercury lamp fed by an a.c. stabilizer. Only the green and yellow mercury lines were used. As light of these wave-lengths is not strongly absorbed by chlorophyll, relatively thick suspensions — giving higher respiration rates and still giving good linearity of the assimilation curves — could be used.

Different light intensities, measured in relative units with the aid of a thermopile-galvanometer system, were obtained by placing Scott NG filters between lamp and vessel. The various light intensities were applied one after the other. Before each measurement of the rate of pressure change (during 5 to 15 minutes) the algae were pre-illuminated during 10 to 15 minutes.

Dark respiration was measured at the start, middle and end of a series of light intensities.

Before each experiment we exposed the algal suspension to strong light during about half an hour; following this procedure the respiration rate was found to be more constant (Fig. 5A.)

In order to get high respiration rates most experiments were performed at 29–30° C. Measurements on leaves were made with small pieces floating in the buffer medium.

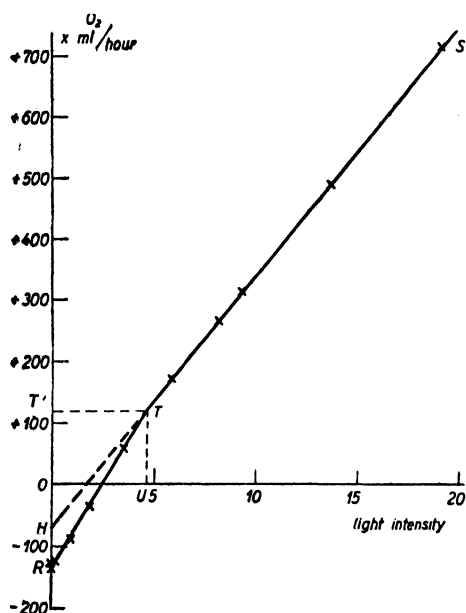


Fig. 2. *Chlorella*, grown in Knop-solution, during 4 days. Exp. 9-4-48

ratio of the slopes of RT and TS being about 1.3. The ratio  $OT^1 : OR$  was found to be fairly constant in various measurements performed with inorganically grown algae of the strain used by us.

References p. 631.

#### EXPERIMENTS

In Fig. 2 a typical curve found with inorganically grown algae is represented. It shows two strictly linear parts, with a sharp bend at T. The production of part ST cuts the ordinate at H, OH being exactly HR (OR represents the magnitude of the dark respiration).  $OT^1$  is about equal to OR, the

Quite other curves were found for algae grown in a medium containing glucose. An example is given in Fig. 3.

In this case we also found two straight lines, but the point of intersection (T) was now situated below the abscissa and point H shifted towards R. It is remarkable in this case that  $T^1H = HR$  and so the slope of RT is exactly twice the slope of TS.

When we interpret TS as photosynthesis (the q quanta process) RT represents really a  $\frac{1}{2}$  q quanta process.

As contrasted with inorganically grown algae the ratio  $OT^1:OR$  varied largely in these experiments.

Experiments were made with algae cultured in media containing diverse amounts of glucose (between 0.01–1.5%), and showing largely varying respiration rates. A distinct correlation could not be detected. Mostly the ratio  $OT^1:T^1R$  varied between 1:1 and 3:1, in some experiments T was found very near to the abscissa or somewhat above it, in one experiment only, no bend was found at all.

In these experiments (more so than it was the case with inorganically grown algae) the decreasing value of the strong dark respiration during the time of measurement proved to be inconvenient.

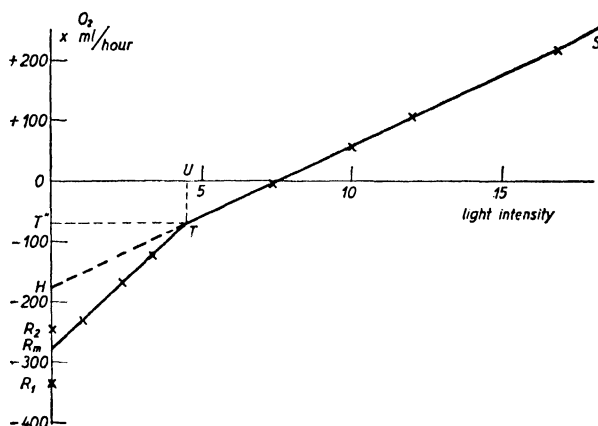


Fig. 3. *Chlorella*, grown in Knop solution + 100 mg glucose per L, during 3 days. Exp. 26-2-48.

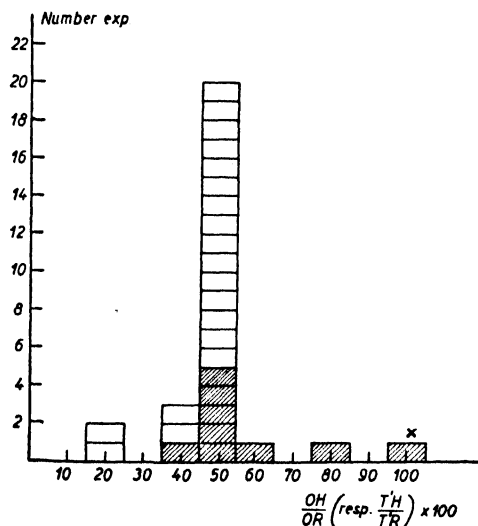


Fig. 4. Classification of results

We see that the position of point T may vary depending upon the conditions. Still more interesting is the question whether such a variability holds for point H too:

In total 28 experiments were made with *Chlorella*.

We estimated for each curve the point of intersection of  $P_1-P_4$  with the ordinate (H Fig. 1) and we computed the value  $\frac{OH}{OR} \times 100$ . If curves were found as represented in Fig. 3, we computed  $\frac{T^1H}{T^1R} \times 100$  (shaded squares in Fig. 4). The argumentation for these different ways of computation will be given on page 629.

The results, classified in groups increasing by 10%, are given in Fig. 4. From the distribution found we may conclude that experimental errors only are responsible for

the finding of other values than 50%. (The experiment marked X, showing no bend at all, was already mentioned above).

This means that the ratio  $\frac{OH}{HR}$  or  $\frac{T^1H}{HR}$  equals 1 under all circumstances.

Finally we made some experiments with two other green plants. With the alga *Haematococcus fluviatilis* (*Volvocales*), grown in an inorganic medium (Knop solution), we found curves of the type represented in Fig. 5. Here we found  $T^1H$  greater than  $HR$ .

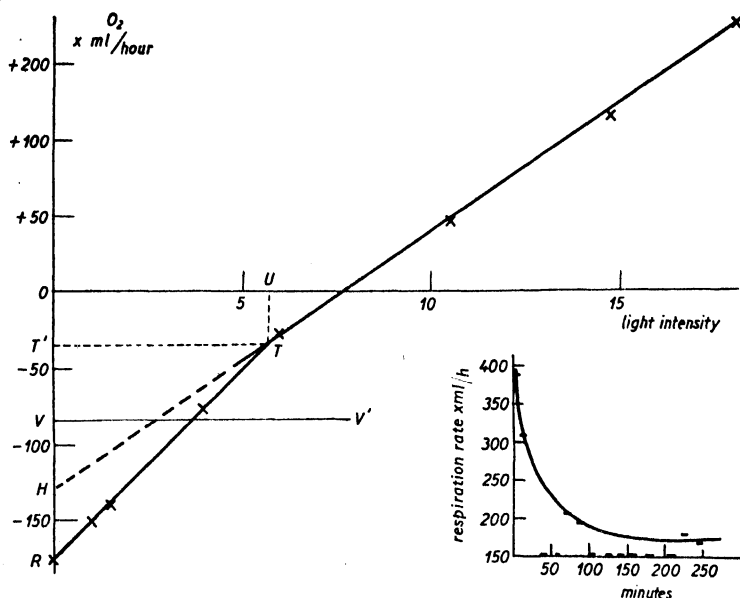


Fig. 5. *Haematococcus*, grown in Knop solution, during 14 days. Exp. 23-3-48. Fig. 5A indicates the decrease of respiration rate with time.

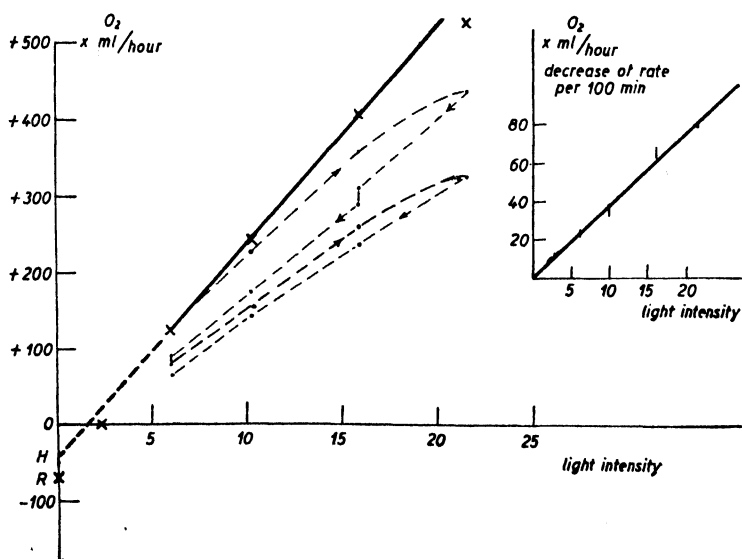


Fig. 6. *Cabomba* leaves . . . . Rates measured one after the other. Decrease fairly proportional with time and with light intensity cf. Fig. 6A. x—x Assimilation curve as computed.

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Fig. 5A shows the strong decrease of the respiration rate with time. By way of interpolation for each light intensity a correction was computed.

Leaves of the submersed aquatic plant *Cabomba*, floating in Warburg buffer showed a decreasing assimilation rate during our experiments (due to the high  $p_H$ ?). As this decrease was fairly proportional with time, it was possible to eliminate this effect to a large extent by measuring with each sample of leaves an assimilation curve several times.

Respiration rates were found to be fairly constant in this case. The light intensity assimilation-curves distinctly showed the effect as represented in Fig. 6, OH being somewhat greater than HR.

No accurate information was obtained regarding the range RT (Fig. 2), although we received the impression that point T was situated as shown in Fig. 2 or at still higher light intensities.

#### DISCUSSION

As we found a perfectly straight line between R and T in the case of *Chlorella* (Fig. 2), and a sharp bend at T, the existence of only one extra process characterized by a different quantum number, seems to be highly probable.

As we invariably found OH (resp. T<sup>1</sup>H) equal to HR, exactly half the amount of light quanta per oxygen molecule evolved (or not respired) is used in this process as compared to photosynthesis. A further indication in this direction is given by the fact that under special circumstances (Fig. 3) the slope of RT was indeed twice the slope of TS, and a higher ratio never occurred.

The position of the transition point T was found to be variable with external conditions and plant material. This is easily explained as a variability of the ratio in which the absorbed quanta are distributed over the two processes running simultaneously at low light intensities.

As RT is a straight line, we must assume that this ratio is exactly constant in the range of light intensities OU (Fig. 2). At U a transition point T is reached and the  $\frac{1}{2}$  q quantum process is saturated. The light absorbed over that at U is used by the q quanta process only, until at very high intensities this latter process also attains saturation.

As can be derived from the slope of the curve represented in Fig. 2, in the range RT  $\frac{2}{3}$  of the light absorbed is used here by the q quanta process and  $\frac{1}{3}$  by the  $\frac{1}{2}$  q quanta process.

In the case represented in Fig. 3 in range RT all light absorbed is used by the  $\frac{1}{2}$  q quanta process, as we will discuss below.

In our previous paper we interpreted the  $\frac{1}{2}$  q quanta process as "light respiration": a direct supply of energy from light quanta to the cell metabolism. (The favourable energetic yield resulting from the omission of the carbohydrate stage occurring in the cycle photosynthesis-respiration.)

We introduced the supposition that simultaneously with the substitution of its function a decrease of the rate of dark respiration occurs. In such a way that the total energy supply of the cell — the sum of the products of both processes is always constant (at least during an experiment). In this picture at point T (Fig. 2) total suppression of dark respiration is reached and the  $\frac{1}{2}$  q quanta process is saturated, providing the

cell with the same amount of energy as the respiration process furnished in the dark.

If we apply this trend of thought to Fig. 3, we have to assume that now part of the dark oxygen uptake ( $OT^1$ ) is *not* suppressed by the  $\frac{1}{2} q$  quanta process, but goes on undisturbed in the light.

It follows from our experiments that deviations from linearity in the assimilation curves at low light intensities are not restricted to *Chlorella*. In leaves of higher plants where next to green cells colourless tissues occur, the latter tissues can be expected to interfere, since in them respiration can hardly be affected by photochemical products in the green cells. So a priori we would expect to find here also a phenomenon as given in Fig. 3, where point H is shifted towards R cf. Fig. 6.

The curve found with *Haematococcus* needs further discussion: Firstly we find a similar shift of point H as with organically grown *Chlorella* cells.

In these cases we have not to deal with different types of cells (coloured or colourless) and we can imagine two possibilities: either there are two types of oxygen consuming processes running in the dark, only one of both being suppressed by light, or the influence of photochemical metabolites is restricted to the neighbourhood of the chloroplast.

Secondly the slope of RT in Fig. 5 indicates a simultaneous occurrence of the two photochemically induced processes; it seems probable that the part of the dark oxygen consumption not influenced by light amounts to OV ( $HV = HR$ ). This computation is suggested by the analogy with the other curves: considering  $VV_1$  as the abscissa we obtain a similar curve as given in Fig. 2.

According to these views we have to accept in this organism the simultaneous occurrence at low light intensities of 4 processes:

1. unsubstitutable dark respiration; 2. substitutable dark respiration; 3. light respiration ( $\frac{1}{2} q$  quanta process); 4. photosynthesis ( $q$  quanta process).

We may accept a localization of the photochemical process (in the chloroplasts), but a distribution of respiration over the whole cell. Then it is indicated by the various curves obtained that the photochemically formed intermediate, base for the  $\frac{1}{2} q$  quanta process, must have a rather long lifetime.

Finally we may point here to investigations of several authors who determined light intensity assimilation curves with leaves as well as with algae. In many cases the relation found at medium intensities was interpreted as a smoothly bent curve, though a straight line, not crossing the origin, fits much better with the measurements plotted (WASSINK *et al.*<sup>2</sup>, KOPP<sup>3</sup>, GABRIELSEN<sup>4, 5</sup>). We like to interpret all these curves in the same way as we did our own ones. The measurements of GABRIELSEN point to the occurrence of the phenomenon described in leaves of higher plants.

#### SUMMARY

The relation between light intensity and rate of photosynthesis was studied at low and medium intensities with suspensions of *Chlorella*, *Haematococcus*, and leaves of *Cabomba*. Various types of curves were found, all showing two strictly linear parts with a sharp bend between.

In the discussion of the various curves we started from the more or less complete suppression of dark respiration by the photochemical reaction.

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## RÉSUMÉ

La vitesse de la photosynthèse a été mesurée relativement aux intensités lumineuses faibles et moyennes avec des suspensions de *Chlorella*, *Haematococcus* et des feuilles de *Cabomba*.

Des courbes d'un caractère divers ont été trouvées, chacune présentant deux portions rectilignes séparées par un coude accentué. Pour la discussion des diverses courbes, nous avons pris pour point de départ la suppression plus ou moins totale de la respiration due à la réaction photochimique.

## ZUSAMMENFASSUNG

Das Verhältnis zwischen Lichtintensität und Geschwindigkeit der Photosynthese wurde bei schwachen und mittleren Intensitäten an Suspensionen von *Chlorella*, *Haematococcus* und an Blättern von *Cabomba* gemessen. Es wurden verschiedenartige Kurven gefunden, die aber alle aus zwei geradlinigen Stücken bestehen. Bei der Besprechung der verschiedenen Kurven gingen wir von der mehr oder weniger vollständigen Ausschaltung der Dunkelatmung durch die photochemische Reaktion aus.

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## AN ANTIGENIC POLYSACCHARIDE, "POLYSACCHARIDE II", ISOLATED FROM TUBERCULIN

by

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Considerable information has been gained concerning a polysaccharide found in tuberculin (LAIDLAW AND DUDLEY, 1925; MUELLER, 1926; RENFREW, 1930; STACEY AND KENT, 1948). It was shown (SEIBERT, PEDERSEN, AND TISELIUS, 1938; TENNENT AND WATSON, 1942) to have a molecular weight of about 7000-9000 to consist of units of mannose, galactose, and arabinose (RENFREW, 1930), and to be ineffective in causing a tuberculin skin reaction (McCARTER AND WATSON, 1942).

Recently we have discovered in tuberculin another polysaccharide with very different properties, and it has been designated "Polysaccharide II" to distinguish it from the former one which has now been called "Polysaccharide I". It is the purpose of this paper to describe some chemical and serological properties of Polysaccharide II.

### EXPERIMENTAL

#### OCCURRENCE

Polysaccharide II was first suspected when we observed in the Tiselius an extra sharp peak with very low electrophoretic mobility on several tuberculin preparations made from a bovine strain (# 523) tubercle bacillus and was, therefore, at first thought to be characteristic of the bovine strain. Since then, however, it has been found in filtrates from three human strains as well as from the B.C.G. strain and, therefore, cannot be considered specific for the bovine type bacillus. Certain tuberculin filtrates have an opalescent appearance which has been shown to be due not to a contaminant but rather to the presence of this polysaccharide, for it was later found that aqueous solutions of the isolated polysaccharide are very opalescent. Moreover, the same strain may yield a tuberculin filtrate containing large amounts of this Polysaccharide II at one time while at other times it is absent. For example, during earlier studies made with the H 37 human strain tubercle bacillus some years ago, the filtrates were clear and none of this polysaccharide was detected, whereas in recent months what is presumably the same strain yields large quantities though it may be significant that during this time the strain has become relatively avirulent. Furthermore, one strain "DT", which is a considerably more virulent human strain, gives a clear filtrate from which only a

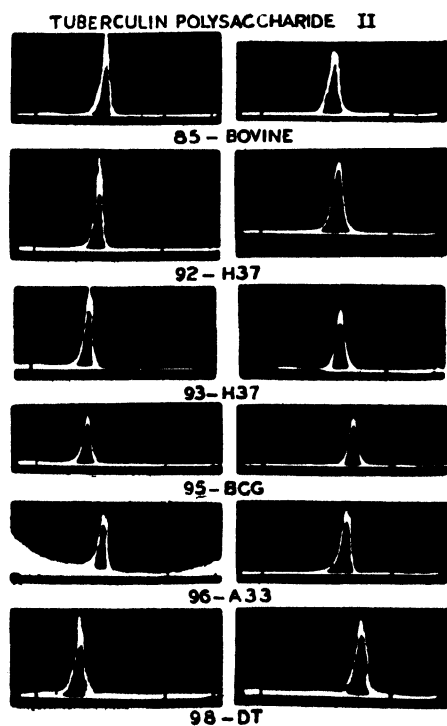
very small amount of the Polysaccharide II can be isolated; an amount so small that it would probably be missed if it were not directly sought by special methods.

## ISOLATION

Polysaccharide II has so far been isolated from five different strains, two bovine, # 523 and B.C.G., and from three human strains, H 37, A 33, and DT. Essentially the same method of isolation was used in all cases, which was as follows:

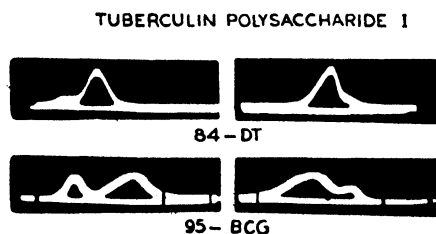
The organisms were grown for 8 to 12 weeks on LONG's synthetic medium and quickly filtered off alive on paper and then passed through a Seitz filter. The filtrates were taken to a cold room maintained at  $1-2^{\circ}\text{C}$  and all further procedures were carried out at this temperature. The sterile filtrates were concentrated by ultrafiltration to about one tenth of their original volume and the filtered solution adjusted to about  $\text{pH}$  4.0 with acetic acid. The resulting precipitates, which consisted mainly of protein C (SEIBERT, 1949), were removed by centrifugation. The supernatants were filtered if not clear, readjusted back to  $\text{pH}$  7.0 with alkali, and sufficient alcohol added to give a concentration of 30%. The resulting precipitates consisted essentially of Polysaccharide II, which formed very opalescent solutions when dissolved in water and gave white powders when dried.

The yields varied considerably with the strain, as mentioned above (see Table I). The bovine # 523, H 37 and A 33 strains yielded significant amounts of this polysaccharide, whereas only small amounts were obtained from the B.C.G. and DT strains.



## CHEMICAL PROPERTIES

*Electrophoresis.* When solutions of the pure isolated polysaccharides were studied by the Tiselius electrophoresis technique in phosphate buffer  $\text{pH}$  7.7,  $\mu = 0.1$ , they were found to have very slow mobilities of  $-1.0$  to  $-1.6 \cdot 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$ , and to consist of a single component only, even after four hours of electrophoresis at a potential gradient of 7 to 9 V per cm (see Fig. 1 and Table I). The peaks remained very sharp on both ascending and descending sides and were in great contrast to the low spreading



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TABLE I  
 ISOLATION AND YIELDS OF POLYSACCHARIDE II

Strain of tubercle bacilli used	LONG's medium used (l)	Age in weeks	Concentrated to ml	Precipitated at pH	Readjusted to pH	Treatment of supernatant	Precipitated with alcohol %	Yield in g	Electrophoretic mobility* ( $10^5 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$ )
85 (Bov.)	40	?	213	3.7	7.0	Pptd with 213 ml in neutral $(\text{NH}_4)_2\text{SO}_4$ . Later treated with charcoal	55	2.46	—1.0
92 (H 37)	11.8	9½	820 (used 790)	4.0	6.5	Concd to 455 ml	20	1.79	—1.4
93 (H 37)	4.5	9	540	4.1	7.0		30	0.86	—1.5
95 (BCG)	26.5	8	1000	4.0	7.0	Concd to 426 ml Pptd with 426 ml neutral $(\text{NH}_4)_2\text{SO}_4$ . Sup. concd Ppt 30% alc. redissolved pH 7.6	30	0.09	—1.5
96 (A 33)	24.5	12	730	4.7 and 3.8			30	0.70	—1.6
98 (DT)	28.7	8½	850	4.4	7.8		30	0.124	—1.6

\* In phosphate buffer pH 7.7 = 0.1

curves found for the Polysaccharide I preparations (see Fig. 2), studied with similar concentrations and conditions.

Even in the presence of other components of tuberculin, Polysaccharide II is clearly distinguishable on the electrophoretic diagrams, due to its sharp peak. An attempt was, therefore, made to determine the  $p_H$  mobility curve in such a mixture,

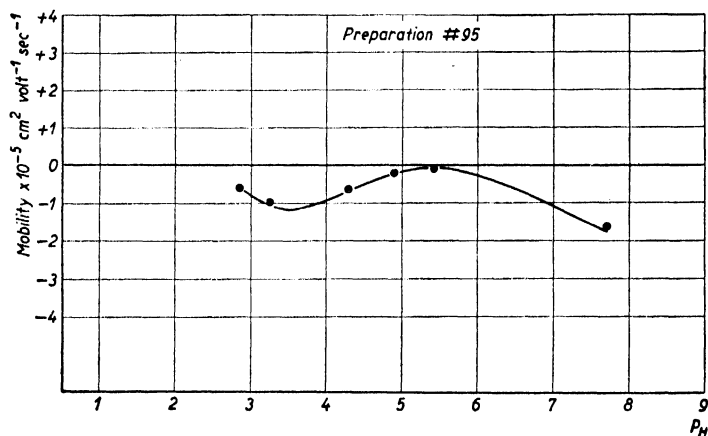


Fig. 3

using citrate, acetate, or phosphate buffers of 0.1 ionic strength. Fig. 3 shows the curve obtained. There was apparently no charge around  $p_H$  5.3, and a slightly acidic charge around  $p_H$  3.5, as well as on the alkaline side of  $p_H$  5.5.

References p. 640.

*Sedimentation and diffusion.* Two sedimentation measurements were made in the SVEDBERG ultracentrifuge on the polysaccharide (Preparation # 85) by Mrs. ELLEN BEVILACQUA at the University of Wisconsin to whom we are very grateful. The resulting constants were  $S_{20} = 2.13$  and  $2.09$ . In both cases the sedimentation curves were symmetrical, indicating considerable homogeneity in the preparation.

Determination by Mrs. BEVILACQUA of the rate of diffusion, using the Lamm diffusion cell, showed a constant of  $D_{20} = 1.0$ . The diffusion curves measured during the experiment showed considerable skewing at the beginning, but definite symmetry at the end. The constants calculated were as follows.

At	55 h	$D_{20} = 2.88$
„	69 „	$D_{20} = 1.3$
„	79 „	$D_{20} = 1.4$
„	96 „	$D_{20} = 1.3$
„	117 „	$D_{20} = 1.1$
„	139 „	$D_{20} = 1.0$

This preparation was the first one isolated, and on electrophoresis showed a very small extra component with slightly higher mobility than the main polysaccharide component. It perhaps was this small contaminant which caused the skewing of the early diffusion curve. Later preparations were entirely free of this component, as was also a repurified polysaccharide (# 85). See Fig. 1.

The sedimentation and diffusion constants would, therefore, suggest a very elongated chain molecule with high molecular weight, of the order of 100000 and having a high dissymmetry ratio.

*Spectrographic absorption.* An 0.45% solution of the polysaccharide # 92 was studied in the Beckmann spectrophotometer in the ultra-violet range and Fig. 4 shows there is no definite absorption, except a small amount at wave length  $\lambda$  2800 Å, indicating possibly the presence of a trace of protein or an amino acid residue. Similar absorption curves have also been obtained with the Polysaccharide I.

*Chemical analyses and structure.* The nitrogen contents of two of the preparations (# 85 and # 92) were determined by the micro-Kjeldahl method and found to be 0.59

and 0.31% respectively. The former result was obtained on the original # 85 preparation, before repurification, and indicates a maximum protein impurity of about 3.3%. For the # 92 preparation it was 1.9%.

*References p. 640.*

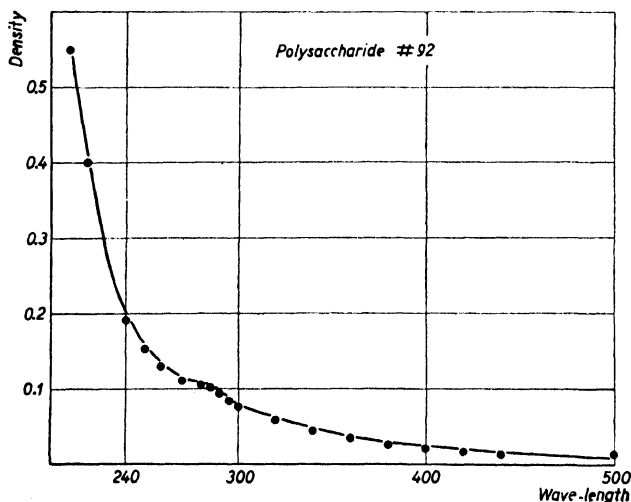


Fig. 4

There was however some evidence from the Ehrlich test that an amino sugar was present.

The diphenylamine reaction for deoxyribonucleic acid was negative when applied to about 25 mg of the repurified # 85 preparation, from which observation and from the spectrographic data, it is safe to conclude that there was no nucleic acid present.

When studied by means of the carbazole reaction (SEIBERT AND ATNO, 1946), a deep pink colour, similar to that shown by glucose, was given, in contrast to the yellowish pink given by the Polysaccharide I preparation. The ratio of the density at wave length  $\lambda$  5400 Å to that at wave length  $\lambda$  4200 Å was 3.97 for preparation # 85 (original) and 5.30 for preparation # 92. These ratios were close to that given by glucose, 4.89, and very different from the ratio of 1.38 for Polysaccharide I, which has been shown (RENFREW, 1930) to consist of mannose, galactose, and arabinose. It would seem, therefore, that Polysaccharide II might consist mostly of glucose units.

Confirmation of this was found in the following chemical studies on preparation # 92. Qualitatively no pentose or uronic constituents could be found. The optical rotation was  $[\alpha]_D^{24} + 165^\circ$  (in water,  $c = 0.2$ ). Upon hydrolysis with 0.1 N sulphuric acid for 6 hours, there was first a slight increase to  $[\alpha]_D^{24} + 171^\circ \rightarrow 186^\circ$ . The polysaccharide was then readily hydrolysed by being heated at  $100^\circ\text{C}$  with N sulphuric acid for 5 hours,  $[\alpha]_D^{24} + 171^\circ$  changing to  $+ 58^\circ$ , a behaviour which resembled that of the polyglucose, dextran.

The mixture of reducing sugars in the hydrolysate was examined chromatographically using the paper-strip technique (PARTRIDGE, 1946). By means of a butanol-ethanol-water mixture as the eluting agent, it was shown that the mixture contained two principal reducing constituents. Comparison of the  $R_f$  values of the two constituents indicated that one of them was glucose, and this was further confirmed by including authentic glucose in the paperstrip chromatogram.

The second slower-moving constituent sugar has not yet been identified.

An attempt was made to separate and characterize constituent sugars of the polysaccharide hydrolysate by the formation of crystalline derivatives. The mixture of reducing sugars obtained as described was treated with ethyl mercaptan in strongly acid solution at  $0^\circ\text{C}$  (WOLFROM AND KARABINOS, 1945). The mixed mercaptals so formed were acetylated in pyridine and acetic anhydride. This yielded two products, one a crystalline substance and the other a syrup (in small quantity). The crystalline diethyl mercaptal pentacetate had m.p.  $44-45^\circ$  alone or with an authentic specimen of glucose diethyl mercaptal pentacetate.

The mixture of reducing sugars from the hydrolysed polysaccharide when refluxed with alcoholic aniline for 4 hours yielded a crystalline anilide, m.p.  $144-145^\circ$  alone or in admixture with an authentic specimen of glucose anilide (IRVINE AND GILMORE, 1906). The presence of glucose as a principal constituent sugar of the polysaccharide was thereby confirmed; it was not possible, by absorption on activated alumina, to identify another sugar from the mother liquors of the glucose anilide.

In an endeavour to ascertain whether the glucose residues in the polysaccharide were linked in the (1:4) positions as in glycogen or starch, specimens of the polysaccharide were incubated at  $35^\circ\text{C}$  with an active  $\beta$ -amylase preparation from soya beans. There was no generation of reducing sugars as measured by the Schaffer-Hartman technique, and therefore it seems probable that the polysaccharide is different from the glycogen or starch type of structure. Moreover, it did not give a reddish brown colour with iodine in contrast to glycogen.

Its properties are being compared with those of dextrans synthesized by various micro-organisms.

#### BIOLOGICAL PROPERTIES

*Skin reactions.* Polysaccharide II (original preparation # 85) when tested intradermally on patients highly sensitive to PPD-S, gave no skin reaction even when administered in a dosage of 0.05 mg, which is ten times the usual second dose of the tuberculin. See Table II.

TABLE II  
SKIN REACTIONS WITH POLYSACCHARIDE II

Patient No.	Area in millimeters of Skin reaction to	
	0.00002 mg PPD-S	0.05 mg Polysaccharide 85
1	36 × 29 × 3	negative
2	24 × 26 × 3	"
3	37 × 33 × 3	"
4	21 × 18 × 2	"
5	12 × 14 × 2	"
6	14 × 17 × 2	"
7	35 × 29 × 3	"
8	31 × 26 × 3	"
9	24 × 22 × 3	"
10	17 × 13 × 2	"
11	48 × 43 × 3	"
12	15 × 19 × 2	"
13	15 × 12 × 2	"

*Precipitin titres.* High precipitin titres were obtained by adding the Polysaccharide II preparations (# 85 and # 92), to the horse antiserum A 5807, from a horse immunized over a long period of time with large quantities of dead tubercle bacilli in the SHARP AND DOHME laboratories. (See bottom of Table III). This horse serum has also given high titres with Polysaccharide I (SEIBERT, 1932, 1944; MASUCCI, McALPINE AND GLENN, 1931), as well as with polysaccharide fractions isolated from the tubercle bacillus (HEIDELBERGER AND MENZEL, 1932, 1937).

Precipitin titres, even as high as 1:50000, were also obtained by using Polysaccharide II with most tuberculous rabbit sera tested, with sera from rabbits vaccinated with B.C.G. and with a few human tuberculous sera (see Table III). The amount of precipitate always was slight with these sera even though some of the titres were high.

Six normal rabbits were sensitized with the original polysaccharide preparation # 85 by eleven weekly intracutaneous injections of 10 mg each. Their sera then were shown to contain antibodies for Polysaccharide II (see Table III) thus demonstrating its antigenicity.

*Immunization.* Six rabbits were given eight intracutaneous injections each of 10 mg. Polysaccharide II (preparation # 85) at weekly intervals and then three successive intraperitoneal injection of the same amount of the polysaccharide to which 10 mg of aluminium hydroxide had been added. The first injection elicited some local reaction evident after 24 hours following the test, and with successive injections the reactions

TABLE III  
PRECIPITIN TITRES WITH POLYSACCHARIDE II

Sera from Name		Condition	Precipitin titrin with Polysaccharide	
			# 85	# 92
Rabbit	108	Normal	0	
"	39	"	0	
"	RB 31	"		0
Rabbit	113	Sensitized with Polys. 85	50 000	
"	109	" " " 85	10 000	
"	K 70	" " " 85	5 000	
"	112	" " " 85	5 000	
"	121	" " " 85	5 000	
"	37	" " " 85	2 000	
Rabbit	40	Spontaneous infection	10 000	100 000
Rabbit	80	Tuberculous	50 000	—
"	81	"	50 000	0
"	171	"	50 000	—
"	172	"	20 000	—
"	40	"	10 000	—
"	41	"	10 000	20 000
"	114	"	4 000	10 000
"	115	"	4 000	1 000
"	99	"	4 000	2 000
"	120	"	20 000	2 000
"	108	"	0	0
"	110	"	0	2 000
"	96	"	—	0
"	105	"	—	5 000?
"	109	"	—	0
"	103	"	—	0
Rabbit	141	Vaccinated with B.C.G.	2 000	
"	121	" " "	5 000	
"	102	" " "	10 000	
"	107	" " "	0	
Rabbit	143	Sensitized with TPA	—	2 000
Human	A.S.	Normal	—	0
"	H.T.	Normal	0	—
"	W.B.	Minimal Tuberculosis	—	0
"	L.W.	Mod. Adv. Tuberculosis	—	50 000
"	F.A.	Far Adv. Tuberculosis	—	20 000
"	R.B.	Far Adv. Tuberculosis	0	
Horse	A 5807	Sensitized with dead TB.	5 000 000	200 000

became larger, even to practically double the size by the eighth injection. Thus evidence of some local sensitization was seen.

Electrophoretic study of the sera of four of these rabbits did not show a great increase in the gamma globulin fraction during these injections.

One week following the eleventh injection these six rabbits, as well as seven controls that received no previous treatment, and six that had been injected three times with a total of 1.25 mg B.C.G., were inoculated intravenously with 0.00001 mg of live Bovine (RAVENEL) strain tubercle bacilli. They were all allowed to live their natural span of life and then at death autopsied. The amount of tuberculosis was noted by evaluating the percentage of total lung tissue involved with tubercles and also the degree of consolidation by the weight of the lungs. Average results for these criteria of the extent of tuberculosis, as well as the average longevity in each group, were recorded in Table IV and indicate that no significant immunity was developed through sensitization with Polysaccharide II.

TABLE IV  
IMMUNIZATION WITH POLYSACCHARIDE II

Method of Treatment	Number Rabbits	Average Longevity in days	Average Tuberculosis in the Lungs	
			Estimated %	Weight H(g)
Controls	7	176	63	52
B.C.G.	6	133	30	46
Polysaccharide # 85	6	175	37	42

#### DISCUSSION

A polysaccharide with a very large particle weight, designated as glycogen, has been isolated from avian tubercle bacilli by CHARGAFF (CHARGAFF AND MOORE, 1944). It lacked any specific biological activity and had a particle weight of about 13.2 million. LAIDLAW AND DUDLEY (1925) also isolated small amounts of glycogen from tubercle bacilli, of the human type. HEIDELBERGER AND MENZEL (1932, 1937), GOUGH (1932) and HAWORTH, KENT AND STACEY (1948) also mentioned the presence of glycogen among the polysaccharides isolated from tubercle bacilli. The Polysaccharide II described in this paper, like the glycogens, gives an opalescent solution in water, and is mainly constituted of glucose units. Nevertheless it does not give a red brown colour with iodine, it has a high biological specificity, shows some antigenicity, more closely resembles the dextrans, and would, therefore, appear to be a newly identified polysaccharide, manufactured in considerable quantity by certain strains of the tubercle bacillus.

Since the question whether or not anything except proteins can serve as antigens is an important one, some caution is necessary. The Polysaccharide II (preparation # 85) used in these studies for sensitization of the rabbits was shown from its nitrogen content to contain a maximum of 3.3% of possible protein, and, therefore, 3.6 mg would be the total protein given with the polysaccharide during the eleven injections. It is hard to believe that this is sufficient protein to account for the elicitation of the precip-

itins obtained. Especially is this true, when 15 injections of a Polysaccharide I preparation which contained even more protein impurity, did not elicit any precipitins. Whether the Polysaccharide II is a part of the cell liberated into the medium or whether it is a true metabolite cannot be determined from the present data.

### SUMMARY

1. A polyglucosan, with a very low nitrogen content, a relatively large particle weight, and forming opalescent solutions in water has been isolated from several strains of human and bovine type tubercle bacilli.

2. Although it consists mainly of glucose, it is not a glycogen but may be similar to a dextran structurally. It is able to elicit precipitins to itself and it gives a high precipitin titre with a horse antiserum and lower titre with some tuberculous rabbit and human sera.

3. It did not induce a significant immunity in a small number of rabbits, nor did it cause a tuberculin reaction in patients who were sensitive to the protein.

### RÉSUMÉ

1. On a isolé de plusieurs souches de bacilles tuberculeux humains et bovins un polyglucosane contenant très peu d'azote et dont les particules sont de poids relativement élevé donnant avec l'eau des solutions opalescentes.

2. Quoiqu'il soit principalement formé de glucose ce n'est pas un glycogène, mais sa structure rappelle quelque peu celle d'un dextrane. Il peut donner naissance à des précipitines et il donne une dilution limite active de précipitine élevée avec l'antisérum de cheval et une dilution-limite active plus faible avec des séra de lapins et humains tuberculeux.

3. Pour un petit nombre de lapins, on n'a pas constaté d'immunité notable; on n'a pas constaté non plus de réactions tuberculines chez les patients qui étaient sensibles à l'action de la protéine.

### ZUSAMMENFASSUNG

1. Ein Polyglucosan wurde von mehreren Stämmen menschlicher und tierischer Tuberkelbazillen isoliert. Der Stickstoffgehalt ist sehr niedrig, das Gewicht der Teilchen ist relativ gross und es gibt mit Wasser opaleszierende Lösungen.

2. Obgleich es hauptsächlich aus Glukose besteht, ist es kein Glykogen, sondern hat möglicherweise eine dextranartige Struktur. Es ist fähig in sich selbst Präzipitin zu formen. Es gibt einen hohen Präzipitin-Titer mit Pferdeantiserum und einen niedrigeren Titer mit einigen tuberkulösen Kaninchen- und menschlichen Seren.

3. Bei einigen Kaninchen wurde keine erhebliche Immunität festgestellt; ausserdem sind keine Tuberkulin-Reaktionen bei den Patienten festgestellt worden, die sich gegenüber Protein empfindlich erwiesen hatten.

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## STUDIES ON THE GLYCOGEN OF *M. TUBERCULOSIS* (HUMAN STRAIN)

by

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There have been frequent observations [LEVENE (1904); HEIDELBERGER AND MENZEL (1937)] regarding the presence of glycogen in numerous microbial cells and various preparations including the well-defined material of CHARGAFF AND MOORE (1944) from the tubercle bacillus have been described.

The present paper is a further contribution to the chemistry of "tubercle glycogen".

### EXPERIMENTAL

#### EXTRACTION WITH ALKALI OF DEGRADED GLYCOGEN FROM *M. tuberculosis*

Moist cells (2000 g) killed by steaming for 3 hours, were extracted exhaustively with acetone in a continuous extractor. During this process, a white crystalline solid (2 g) accumulated, apparently by sublimation in the condenser of the apparatus even when CO<sub>2</sub> was completely excluded. The substance was identified chemically as ammonium carbonate and this was confirmed by X-ray powder photographs. The acetone-treated cells were then extracted exhaustively with ether. The defatted organisms (210 g) were extracted with a solution of sodium hydroxide (1 N, 2 l) for 24 hours at 90° C. The insoluble residue was removed and the liquor acidified with acetic acid. On addition of ethanol (4 vols) a precipitate of crude carbohydrate material was obtained. This was redissolved in a little dilute acetic acid ( $\frac{N}{20}$ ) and dialysed for 48 hours. After centrifuging the polysaccharide (15 g) was reprecipitated with excess ethanol and was dried by washing with ethanol and ether. This product had  $[\alpha]_D^{19} + 52^\circ$  (in water), Ash 55%; it gave a strongly positive Dische and Molisch tests, and gave a reddish-brown coloration on addition of dilute iodine solution.

#### FRACTIONATION OF DEGRADED GLYCOGEN

The impure carbohydrate (10 g) was separated into fractions by dissolving the crude product in water (100 g) and adding ethanol dropwise to the stirred solution until some precipitation occurred. The fraction was separated and more ethanol cautiously added to the supernatant to give a further precipitation. In this way, a series of fractions were obtained as shown in Table I.

It was clear that the glycogen was contained almost wholly in fractions 2 and 3. These were combined with other iodine-staining fractions and refractionated by alcoholic precipitation as before (Table II). The final material, Fractions 11 and 12, gave a strong red coloration with iodine and were used for the investigation.



TABLE I  
FRACTIONATION OF CRUDE CARBOHYDRATE (10 g)

Fraction obtained by adding ether and ethanol	Weight of precipitated g	$[\alpha]_D^{19*}$	Bial test	Dische test	Iodine coloration	Polysaccharide
1	0.05	+20°	—	—	—	Mainly inorganic
2	0.23	+67°	—	±	brown	Impure degraded bacterial glycogen
3	0.42	+85°	+	±	reddish-brown	
4	2.8	+86°	++	+++	—	Specific polysaccharide (HAWORTH, KENT, AND STACEY, 1948a)
5	0.68	+72°	++	+	—	
6	0.18	+58°	++	+	—	Mixed Fractions
7	0.45	+40°	++	+	—	
8	4.0	+30°	++	++	—	Specific Polysaccharide (HAWORTH, KENT, AND STACEY, 1948b)
9	1.51	+27°	++	++	—	
10	0.23	+35°	+	±	—	Not examined

\* Corrected for ash.

TABLE II  
FURTHER FRACTIONATION OF DEGRADED GLYCOGEN

Fraction No.	Amount of ethanol added (ml)	$[\alpha]_D^{20}$ in water	Weight g	Iodine coloration	Dische test	Polysaccharide
11	6	+168°	0.25	++	—	Degraded bacterial glycogen
12	10.2	+171°	0.46	++	—	
13	13.0	+100°	0.21	±	—	Mixture
14	15.2	+89°	0.08	—	—	Somatic polysaccharide
51	4	+78°	0.09	—	±	Somatic polysaccharide
16	25	+52°	0.10	—	±	Somatic polysaccharide

Material corresponding to Fractions 11 and 12 was collected and purified further by repeated fractional precipitation from solvents, dialysis, etc., until an essentially hydrogenous, electrophoretically immobile product,  $[\alpha]_{\text{D}}^{20^\circ} + 178^\circ$  was obtained. It gave a typical "glycogen" stain with iodine.

#### HYDROLYSIS WITH DILUTE MINERAL ACID

The polysaccharide (15 mg) dried for 4 hours at  $60^\circ$ , was dissolved in sulphuric acid (0.5 ml of 0.5 N), and heated at  $100^\circ\text{C}$ . The hydrolysis was complete in 4 hours ( $[\alpha]_{\text{D}}^{18^\circ} + 178^\circ \rightarrow + 58^\circ$  equilibrium value).

#### PAPER CHROMATOGRAPHY OF THE HYDROLYSED POLYSACCHARIDE

The hydrolysate from 15 mg of glycogen was made neutral with barium carbonate, filtered and concentrated to dryness. The residue was taken up in two drops of water and a specimen of this solution was transferred (by a capillary tube) to a strip of Whatman No. 1 filter paper (10 cm.  $\times$  30 cm). Alongside was placed a similar amount of a 2% solution of glucose in water. The chromatography was carried out over 12 hours at room temperature using butanol (40%)/ethanol(10%)/water(50%).

The chromatogram, after development with dilute ammoniacal silver nitrate solution showed clearly the presence of glucose only.

#### ISOLATION OF GLUCOSE ANILIDE

The remaining neutralized hydrolysate (see above) was dried and refluxed for 2 hours with dry ethanol (2 ml) containing 10 mg of aniline. After filtration, a solid separated which after recrystallisation had m.p.  $145-6^\circ$  alone or in admixture with authentic specimen of D-glucose anilide.

#### REDUCING SUGAR CONTENT

The polysaccharide (2.0 mg) was hydrolysed by  $\frac{\text{N}}{2}$   $\text{H}_2\text{SO}_4$  (1 ml) at  $100^\circ\text{C}$  for 4 hours, neutralized ( $\text{p}_\text{H}$  8) with sodium carbonate and the amount of reducing sugar estimated by titration with Schaffer-Hartmann reagents, giving 2.17 mg of glucose, *i.e.*, 98% reducing sugar.

##### 1. *Attempted Amyolytic degradation of the degraded glycogen*

The dried glycogen (2.5 mg) dissolved in 0.5 ml of phosphate buffer at  $\text{p}_\text{H}$  7 was treated with 0.5 ml of salivary amylase at  $25^\circ\text{C}$ . After 35 minutes the iodine coloration had completely disappeared and the resulting solution was reducing to Fehling's solution. Identical results were obtained with  $\beta$ -amylase from soya beans.

##### 2. "Starter" function (SITCH AND PEAT-private communication)

The polysaccharide (5 mg) was dissolved in 1 ml of citrate buffer at  $\text{p}_\text{H}$  6, containing 1 ml of 0.1 M potassium glucose-1-phosphate and this solution was treated with 1 ml of a purified potato phosphorylase (P-enzyme) for 12 minutes at  $35^\circ\text{C}$ . After removal,

*References p. 647.*

of the protein with trichloroacetic acid (5 ml 6%) the inorganic phosphorus liberated was estimated colorimetrically.

Phosphorus liberated = 7.4 (mg/100 ml)

Activating power ratio = 0.22 =  $\frac{\text{mg/ml phosphorus liberated by 5 mg polysaccharide}}{\text{mg/ml phosphorus in control}}$

#### ISOLATION OF GLYCOGEN BY MEANS OF TRICHLOROACETIC ACID

Moist organisms (200 g) were ground with trichloroacetic acid (0.1 N; 200 ml) for 17 hours at room temperature. The cell debris was removed by centrifuging and by passing the resulting liquid through a Seitz filter. The filtrate was dialysed in cellophane against running water for 64 hours. After being centrifuged, the solution was then concentrated by evaporation to 10 ml. This solution was dialysed for a further 24 hours and finally it was freeze-dried.

The product (80 mg) gave negative tests for protein and nucleic acid, but imparted the characteristic coloration with iodine and gave a strongly positive Molisch test. The optical activity could not be determined initially due to the opalescence of the solution.

% N = 0.6 after precipitation of the polysaccharide from aqueous solution by addition of alcohol (3 vols).

The substance underwent hydrolysis readily with dilute mineral acid  $\frac{N}{2}$   $\text{H}_2\text{SO}_4$   $[\alpha]_D^{20} + 92^\circ$  (after 10 minutes),  $+ 57^\circ$  (after  $5\frac{3}{4}$  hours) ( $c = 0.5$ ). The resulting solution, after neutralization was concentrated and examined chromatographically on filter paper. As before, only one component, glucose appeared and this had  $R_F$ , 0.187.

The fraction was examined by the method described for any possible "starter" activity. Under the conditions of the experiment, there was no detectable liberation of phosphorus.

#### POTASSIUM PERIODATE OXIDATIONS

##### a) On degraded glycogen

37.35 mg of the fractionated polysaccharide were dissolved in 50 ml of 10% potassium chloride to which was added 5 ml of sodium metaperiodate (0.494 M). The mixture was shaken and allowed to stand at room temperature.

At intervals of 24 hours, 5 ml portions were withdrawn, diluted with a little distilled water and 0.1 ml of ethylene glycol added. The formic acid liberated was titrated with 0.006 M sodium hydroxide using methyl red as the indicator.

The liberation of acid appeared to be complete after 140 hours.

100 mg glycogen = 1.72 ml 0.006 N NaOH, thus

3 mols formic acid = 1870 g polysaccharide  
= 11.6 glucose units

##### b) On NaOH extracted glycogen (unfractionated material)

0.03735 g = 0.64 ml · 11 ml 0.0065 N NaOH

Therefore 3 mols formic acid =  $\frac{0.03735 \cdot 3 \cdot 1000}{0.64 \cdot 11 \cdot 0.0065 \cdot 162} = \underline{17.9}$  glucose units

## TRICHLOROACETIC ACID EXTRACTED GLYCOGEN

0.02789 g dissolved in 50 ml 10% KCl + 5 ml 0.4 M NaIO<sub>4</sub>

After 140 hours

$$0.02786 \text{ g} = 0.48 \text{ ml} \cdot 11 \text{ ml } 0.0065 \text{ N NaOH}$$

$$\text{Therefore 3 mols formic acid} = \frac{0.02786 \cdot 3 \cdot 1000}{0.48 \cdot 11 \cdot 0.0065 \cdot 162} = \underline{15.1} \text{ glucose units}$$

## DISCUSSION

The presence of a polysaccharide, which in solution gave a coloration with iodine similar to that given by glycogen, was described by LEVENE (1904) who obtained it by extraction of *M. tuberculosis* cells with dilute sodium chloride solution. Further mention of such a carbohydrate was made by WARKANY (1925) and a method was devised by LAIDLAW AND DUDLEY (1925) for separating it from the pentose-containing specific polysaccharides of the organism. These workers showed that on acid hydrolysis of the polysaccharide glucose only was produced.

Later workers (CHARGAFF AND SCHAEFER, 1935; HEIDELBERGER AND MENZEL, 1937) have reported the presence of this "glycogen" and have employed salivary enzymes for removing it from the other carbohydrates of the organism. Further references to this polysaccharide are given in a recent review by KENT AND STACEY (1948).

A detailed examination of the "glycogen" of the avian strain of *M. tuberculosis* has been carried out by CHARGAFF AND MOORE (1944). Their product was isolated from the defatted organism by use of a borate buffer and also by the well established method of extraction of the cells with a dilute solution of trichloroacetic acid. The glycogen which gave readily the characteristic coloration with iodine, appeared to be in a relatively undegraded state since an examination by the electrophoretic and ultracentrifuge methods showed that it had a very high particle weight, and an axial ratio of 11. The authors showed furthermore that the glycogen after mild acid hydrolysis gave a reducing value of 102% calculated as glucose. The evidence suggested that the carbohydrate was indeed a true glycogen having a complex structure. No chemical evidence was provided concerning the length of the chains or their degree of branching.

CHARGAFF (1947) has reported further that this glycogen fraction was degraded by  $\beta$ -amylase but did not itself behave as a "starter" in the synthesis of glycogen from glucose-1-phosphate by the action of muscle phosphorylase (CORI, 1939), although after degradation by dilute alkali, the glycogen was able to function as a starter. It is known that only small chains of -1:4-linked polyglucosans function as starters either with muscle phosphorylase or with potato phosphorylase (BOURNE, PEAT, AND SITCH, private communication).

No immunological function has yet been ascribed to any glycogen fraction from any strain of *M. tuberculosis*.

In the course of investigation of the carbohydrate constituents of the cells of *M. tuberculosis* (human strain) (HAWORTH, KENT, AND STACEY, 1948) it was found that a carbohydrate giving the characteristic red staining reaction with iodine, was obtained by extraction of defatted cells with dilute alkali. The substance was separated from the contaminating specific polysaccharides and nucleic acids by fractional precipitation by alcohol from an aqueous solution. The purified carbohydrate was considered to be a true

glycogen because of the following properties: a) in solution, it possessed the characteristic red staining property of a glycogen, b) it gave typically opalescent aqueous solutions and had  $[\alpha]_D^{20} + 178^\circ$  in water, c) hydrolysis proceeded rapidly with dilute acid  $[\alpha]_D^{20} + 178^\circ \rightarrow [\alpha]_D^{20} + 58^\circ$  forming a reducing syrup, in which glucose was identified as the sole constituent, d) the substance was completely degraded by  $\beta$ -amylase to a reducing sugar, e) it behaved as a starter in a phosphorylase synthesis.

The stages in the purification of the glycogen were followed polarimetrically and by the chromatographic examination on paper of hydrolysed test fractions of the substance (PARTRIDGE, 1946, 1947). By the latter method, the hydrolysate of the final purified glycogen showed only one constituent, having  $R_F$ , 0.186 in a butanol-ethanol-water system ( $R_F$  0.18 for glucose under the same conditions). Estimation of the total reducing sugar by the SCHAFFER-HARTMANN method in this hydrolysate gave a value 98% calculated as glucose.

The presence of glucose was confirmed by the isolation of crystalline glucose anilide from the hydrolysed polysaccharide.

The optical rotation and rate of hydrolysis of the final glycogen fraction were in close accord with the corresponding data given by glycogen fractions from other sources.

Enzymatic degradation proceeded rapidly at  $37^\circ\text{C}$  using  $\beta$ -amylase from saliva or Soya beans. The starter function was determined using potato phosphorylase and potassium glucose-1-phosphate at  $35^\circ\text{C}$ . Controls carried out simultaneously were negative in all cases.

Attempts to demonstrate the presence of a phosphorylase in the culture filtrate of the human strain organism at various stages of growth were unsuccessful.

Oxidation of the glycogen at room temperature with potassium periodate using HIRST's (1945) extension of BARRY's (1942) method was of some interest. By this technique, each glycogen chain (assuming that there is no branching of the chain) gives rise to three moles of formic acid. Thus, the chain length of the glycogen under consideration appeared to consist of 12-glucopyranose units. Satisfactory controls were carried out with glycogen fractions isolated from guinea pig liver and rabbit liver.

For comparison, a further glycogen fraction has been isolated from the human strain organism using a method analogous to that of CHARGAFF AND MOORE (*loc. cit.*) namely by the extraction of defatted organisms with dilute trichloroacetic acid. By this means a substance was obtained, which after reprecipitation by alcohol from aqueous solution was nitrogen free, and had the characteristic staining property with iodine. In aqueous solution, the high degree of opalescence prevented the satisfactory measurement of its optical rotation. After hydrolysis with dilute mineral acid, the resulting solution  $[\alpha]_D^{20} + 60^\circ$  was strongly reducing to Fehling's solution, and showed only a single component reducing sugar when examined by paper chromatography. The initial fraction in this case did not behave as a starter in phosphorylase synthesis.

Oxidation of this fraction with potassium periodate gave results similar to those given by the alkali-extracted material though it differed in having a chain-length of 15 glucose units. It would appear that the glycogen isolated by the trichloroacetic acid method is a more complex macromolecule than the alkali-extracted material.

#### SUMMARY

A description is given of the isolation of a degraded glycogen from the mixed polysaccharide of *M. tuberculosis* human strain and it would appear to possess the usual chain units of 10-12 glucose residues.

References p. 647.

## RÉSUMÉ

On décrit l'isolation d'un glycogène dégradé à partir du mélange de polysaccharides de souche humaine de *M. tuberculosis*, lequel paraît posséder les chaînes habituelles de 10-12 résidus de glucose.

## ZUSAMMENFASSUNG

Die Isolierung eines degradierten Glykogens aus dem gemischten Polysaccharid von menschlichem *M. tuberculosis* wird beschrieben. Es scheint die übliche Ketteneinheit von 10-12 Glucose-resten zu enthalten.

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## SUR LA FORMATION DE LA THYROXINE ET DE SES PRÉCURSEURS DANS LES IODOPROTÉINES ET LES IODOPEPTONES. II

par

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### INTRODUCTION

La formation de monoiodotyrosine, de diiodotyrosine et de thyroxine par action de l'iode sur les protéines a été démontrée qualitativement par LUDWIG ET VON MUTZENBECHER<sup>1</sup> et nous l'avons étudiée quantitativement en fonction du nombre d'atomes d'halogène mis en œuvre par molécule de tyrosine<sup>2</sup>. La principale conclusion se dégageant de nos résultats est la présence dans les protéines de certains restes de tyrosine aptes à se condenser pour donner naissance à la thyroxine par ioduration totale, tandis que d'autres y demeurent alors à l'état de diiodotyrosine. Comme l'avaient antérieurement observé RFINEKE ET TURNER<sup>3</sup>, et comme nous l'avons confirmé, un optimum de rendement en thyroxine se manifeste lorsque l'on fait agir six atomes d'iode par molécule de tyrosine, un excès d'halogène provoquant la dégradation partielle du produit initialement formé. Or, la valeur du rapport: tyrosine % de la protéine non iodée/thyroxine %, ne dépasse pas alors 0.23 pour la caséine et 0.20 pour la thyroglobuline iodées. Il est donc probable qu'une égale fraction de la tyrosine totale des deux protéines — le sixième environ — participe à la réaction donnant naissance à la thyroxine. Tel n'est plus le cas pour la fibroïne de la soie, dont PITT RIVERS ET MICHEL<sup>4</sup> ont montré que l'ioduration comporte un rendement en thyroxine de 0.4%, malgré une teneur initiale en tyrosine égale à 12.0%, la caséine et la thyroglobuline renfermant respectivement 7.2 et 3.3% de cet acide aminé. Néanmoins, il était possible que ce fait soit particulier aux scléroprotéines, puisque, comme l'ont récemment établi deux d'entre nous, les gorgonines renferment en abondance de la monoiodotyrosine et de la diiodotyrosine, mais seulement des quantités minimales de thyroxine<sup>5</sup>. Il y avait dès lors intérêt à étendre nos recherches antérieures à d'autres protéines solubles de teneurs diverses en tyrosine. Le premier but de ce travail a été d'étudier la formation des dérivés iodés de celle-ci dans la zéine et dans l'insuline.

Par ailleurs, si la réactivité de la tyrosine présente dans les protéines est liée à la structure de celles-ci, certains restes de cet acide aminé doivent y occuper une position privilégiée leur permettant de se condenser pour donner naissance à la thyroxine. LERMANN ET SALTER<sup>6</sup>, RAUCH<sup>7</sup> ont montré que l'hydrolyse alcaline ou enzymatique partielle de la thyroglobuline et d'iodoprotéines artificielles libère des peptones, dont certaines renferment, en tant qu'acide aminé iodé, les unes de la thyroxine, les autres de la diiodotyrosine. Tout se passe donc comme si la première était localisée dans des

régions des molécules protéiques où les restes de tyrosine qui lui donnent naissance occupent initialement une position particulière permettant sa condensation. Dès lors, on pouvait penser que l'hydrolyse partielle d'une protéine avant ioduration serait susceptible de libérer avec une vitesse inégale les molécules de tyrosine présentes, les unes à l'extrémité, les autres au sein de chaînes peptidiques, et que la réactivité de cet acide aminé serait très différente dans une protéine et dans ses peptones. Aussi le second but de ce travail a-t-il été d'étudier la formation de la thyroxine à partir de peptones peptiques de caséine, protéine dont le comportement à cet égard nous était déjà connu<sup>2</sup>.

Un problème complémentaire du précédent a été abordé afin d'étendre nos recherches sur un autre plan. L'activité biologique des protéines thyroïdiennes est proportionnelle à leur teneur en "iode acido-insoluble" (thyroxinien) (PARKES<sup>8</sup>), alors qu'il n'en est pas ainsi pour les iodoprotéines artificielles (DEANESLY ET PARKES<sup>9</sup>). Or, REINEKE, WILLIAMSON ET TURNER<sup>10</sup>, étudiant les conditions dans lesquelles l'ioduration de diverses protéines leur confère une activité, ont montré que celle-ci se développe au cours d'une incubation de 20 heures à 70° après un bref temps d'action de l'halogène (2 heures environ) à 37°. Nous avons étudié dans un premier mémoire la formation de la thyroxine et de ses précurseurs en présence de quantités croissantes d'iode. Il a paru utile d'acquérir en outre des connaissances sur l'évolution de ce processus en fonction du temps d'action de la quantité optima d'halogène.

L'objet de ce travail est donc d'étudier la formation de la thyroxine et de ses précurseurs par ioduration de la zéine et de l'insuline, d'une part, de peptones de caséine, d'autre part, et son évolution dans le temps.

#### PARTIE EXPÉRIMENTALE

1. *Ioduration de la zéine et de l'insuline.* — L'halogénéation de la zéine a été réalisée par NEUBERGER<sup>11</sup> en faisant réagir avec cette protéine 5% de plus de la quantité d'iode théoriquement nécessaire pour transformer la tyrosine en dérivé diiodé. Le produit obtenu renferme 7.54% I (théorie: 7.65%) et le déplacement de  $pK_{OH}$  sur sa courbe de titration montre qu'il n'existe plus de tyrosine dans l'iodoprotéine, laquelle ne donne pas la réaction de MILLON. Il convenait pour nos recherches d'opérer l'halogénéation au moyen de quantités croissantes d'iode agissant sur divers échantillons de zéine. Nous l'avons réalisée par la technique suivante\*. Des solutions à 2.5% de zéine purifiée (N = 15.29%, tyrosine = 5.84%, cendres = 0.10%) dans l'éthanol à 85° additionné de 5% d'ammoniaque (d = 0.925) ont été traitées par des quantités de solution N d'iode correspondant de 1 à 8 atomes I par molécule de tyrosine. Les opérations sont conduites à 0°. Après 1 heure de contact, le mélange est porté à  $p_H$  = 6.9 et versé dans 4 volumes de solution 0.035 M de phosphates mono- et dipotassique (mélange équimoléculaire, de  $p_H$  = 6.7), ce qui provoque la précipitation de l'iodozéine. On purifie cette dernière par solubilisation dans l'éthanol à 90° et précipitation aqueuse, jusqu'à disparition des ions  $I^-$  adsorbés, ce qui exige au moins deux séries d'opérations. Les préparations, lavées rapidement à l'éthanol, ont été séchées sous vide. Elles se présentent comme des poudres faiblement colorées (chamois) dont les caractères de solubilité sont analogues à ceux de la zéine.

\* Nous remercions le Professeur NEUBERGER (*National Institute for Medical Research, Hampstead, London, N.W. 3*) d'avoir mis à notre disposition la zéine utilisée dans ces recherches.

*Bibliographie p. 657.*



L'ioduration de l'insuline par HARINGTON ET NEUBERGER<sup>12</sup> a conduit ces auteurs à l'obtention d'une préparation renfermant 15.4% I, soit environ le taux correspondant à la transformation totale de la tyrosine (12.5%) en diiodotyrosine (valeur théorique: 16.37% I). L'insuline étant soluble dans l'eau, nous l'avons traitée par la technique de REINEKE ET TURNER, celle employée par HARINGTON ET NEUBERGER donnant naissance à un produit biologiquement inactif. En raison de la rareté de cette protéine, nous nous sommes bornés à l'halogéner en présence de 6 atomes d'iode par molécule de tyrosine\*. La technique employée a été la suivante: On dissout dans un tube à centrifuger de 35 ml 0.500 g d'insuline cristallisée (tyrosine: 12.2%) dans 17 ml d'eau distillée. On ajoute 0.125 g CO<sub>2</sub>NaH et une goutte NaOH N pour réaliser un p<sub>H</sub> très voisin de 8.0. On porte au bain-marie à 37° et l'on ajoute, en deux heures et par petites fractions, 0.275 g d'iode finement pulvérisé, le p<sub>H</sub> étant maintenu à 8.0 au moyen de NaOH 0.5 N et l'homogénéité du milieu étant assurée par une agitation mécanique. L'addition d'iode terminée, on porte à 70°, on ferme au moyen d'un joint à mercure traversé par un agitateur et l'on poursuit le chauffage pendant 20 heures, sous agitation continue. Après refroidissement, on acidifie à p<sub>H</sub> = 5.3 par l'acide acétique à 10%, ce qui provoque la précipitation de l'iodoprotéine. On centrifuge, on met en suspension le culot dans 20 ml d'eau distillée et, après avoir acidifié à p<sub>H</sub> = 3.0 avec de l'acide chlorhydrique N on porte au bain-marie à 45°. La protéine dissoute est alors précipitée à p<sub>H</sub> = 4.5 par addition de NaOH 5 N et séparée par centrifugation. Le précipité, mis en suspension dans 20 ml d'eau distillée, est dissous par addition de NaOH N jusqu'à p<sub>H</sub> = 9.0, précipité par l'acide chlorhydrique N à p<sub>H</sub> = 4.5, redissous à p<sub>H</sub> = 9.0 et séparé à p<sub>H</sub> = 4.5. Ces opérations ont pour but d'éliminer les iodures adsorbés en abondance lors de la première précipitation. Elles ont permis de recueillir 0.470 g d'une protéine faiblement colorée (chamois), renfermant 14.8% I et ne donnant pas la réaction de MILLON.

Les iodozéines et les iodoinsulines ont été analysées en ce qui concerne leurs teneurs en iode total (méthode de LEIPERT<sup>13</sup>), en tyrosine (méthode de LUGG<sup>14</sup>), en monoiodo-

TABLEAU I

TENEURS EN IODE TOTAL, EN TYROSINE, EN MONOIODOTYROSINE, EN DIIODOTYROSINE ET EN THYROXINE D'INSULINE ET DE ZÉINES IODÉES A DIVERS DEGRÉS

Nombre d'atomes I mis en œuvre par mol de tyrosine	I total %	Tyrosine %	Monoiodotyrosine %	Diiodotyrosine %	Thyroxine %
A. INSULINE					
0 (témoin)	—	12.20	—	—	—
6	14.80	0	0	(non dosé)	1.35
B. ZÉINE					
0 (témoin)	—	5.84	—	—	—
1	1.21	4.20	2.25	traces	traces
2	2.10	3.30	4.35	1.05	0.20
4	4.50	1.10	3.50	3.20	0.65
6	6.85	0.86	2.30	5.65	1.61
8	9.70	0.50	0.81	6.21	1.50

\* Trois échantillons d'insuline cristallisée ont été soumis à l'ioduration. Nous remercions les Professeurs J. C. DRUMMOND (*Nottingham*), E. JORPES (*Karolinska Institutet, Stockholm*) et K. LINDERSTRÖM-LANG (*Carlsberg Laboratorium, Copenhagen*) et les Maisons BOOTS, LEO et VITRUM, qui ont bien voulu nous les adresser. Ces trois préparations présentaient la même teneur en tyrosine (12.2%).

tyrosine, en diiodotyrosine et en thyroxine (méthode de ROCHE ET MICHEL<sup>15</sup>). On trouvera dans le Tableau I les résultats obtenus.

Ces données seront discutées plus bas. Le fait le plus significatif découlant de leur examen est que le rendement en thyroxine de l'ioduration de la zéine est sensiblement supérieur à celui de l'insuline, bien que cette dernière, soit plus de deux fois plus riche en tyrosine. Par ailleurs, comme dans le cas des protéines étudiées antérieurement, la thyroxine ne prend naissance qu'en présence de quantités importantes d'iode. Le meilleur rendement en cet acide aminé est obtenu par action de 6 atomes I par molécule de tyrosine et les produits ainsi préparés sont d'autant plus pauvres en monoiodo-tyrosine qu'ils sont plus riches en halogène.

2. *Ioduration de peptones pepsiques de caséine.* — La fixation de l'iode par les peptones est depuis longtemps connue, mais la nature des combinaisons halogénées présentes dans leurs dérivés n'a pas été précisée à notre connaissance. Ayant antérieurement étudié les modalités de la formation des acides aminés iodés dans la caséine, nous nous sommes proposés d'halogéner des peptones pepsiques de cette protéine, afin de comparer leur composition en dérivés halogénés de la tyrosine à celle d'iodocaséines.

Six échantillons de peptone ont été préparés par le procédé suivant. 10 g de caséine de Vache pure (LINDERSTRÖM-LANG) (N = 15.62%, cendres = 0.1%, tyrosine = 7.20%) sont triturés au mortier dans 50 ml d'eau distillée et dissous par addition de 8 ml NaOH N. On complète à 150 ml avec de l'eau distillée et l'on ajoute, par petites portions, un mélange de 128 ml d'eau et de 22 ml HCl N, puis 0.3 g de pepsine dissous dans 50 ml HCl N/10. Après avoir ajusté le  $p_H$  à 1.5 et saturé le milieu de toluène, on porte à 37°, en ayant soin de contrôler le  $p_H$  chaque jour et, si besoin est, de le ramener à  $p_H$  = 1.5 après chaque vérification. Au bout de trois jours, on concentre sous vide à 25 ml à une température inférieure à 37° et l'on amène à  $p_H$  = 5.7 au moyen de NaOH N. La tyrosine libérée par la pepsine précipite en 24 heures à 0°; on la sépare par centrifugation et le liquide surnageant est ensuite additionné de 4 volumes d'éthanol à 95°, ce qui provoque la formation d'un précipité de peptone, que l'on centrifuge et sèche sous vide. Le rendement de ces opérations est d'environ 1 gramme.

Après avoir dosé la tyrosine dans le produit obtenu, on procède à son ioduration par la technique de REINEKE, WILLIAMSON ET TURNER<sup>10</sup>. Pour cela, on ajoute, en 2 heures et par petites portions, 0.109 g d'iode finement pulvérisé à 2.6 g de peptone dissous dans 70 ml d'eau renfermant 0.64 g de bicarbonate de sodium, la température étant maintenue à 37°. Cette quantité d'iode correspond, dans l'exemple choisi, à 5.7 atomes I par molécule de tyrosine. Le  $p_H$  du milieu est maintenu à voisinage de 7.8 pendant cette opération, que l'on fait suivre d'un chauffage de 20 heures à 70°, sous agitation continue, dans un récipient fermé par un joint à mercure. Après refroidissement, on complète le volume à 80 ml et l'on dose l'iode combiné et la thyroxine dans des prises d'essai. La teneur de la peptone en iode combiné est calculée au moyen de la différence: I total % (LEIPERT)—I des iodures<sup>16</sup> et le taux de la thyroxine déterminé par colorimétrie<sup>15</sup> après hydrolyse barytique. La solution (iodopeptone totale) a été fractionnée par addition de six volumes d'éthanol à 95° (mélange hydroalcoolique à 81.5° d'éthanol) en un précipité que l'on isole par centrifugation et un produit soluble que l'on recueille après évaporation sous vide. L'analyse de l'iodopeptone totale et de ses deux fractions a donné, sur une série de préparations, des résultats rassemblés dans le Tableau II.

Les peptones de caséine étudiées, toutes beaucoup plus pauvres en tyrosine que

TABLEAU II  
TENEURS EN IODE TOTAL, EN THYROXINE ET EN DIIODOTYROSINE DE DIVERSES IODOPEPTONES DE CASÉINE

N° et teneur en tyrosine de la peptone non iodée initiale	Atomes I mis en œuvre p. molécule de tyrosine	Thyroxine % de l'iodopeptone totale	Pourcentage de l'iodopeptone insoluble dans l'éthanol à 81.5° en			Thyroxine % de l'iodopeptone sol. dans l'éthanol à 81.5°
			iode	thyroxine	diiodotyrosine	
(I) 1.00	6.4	—	1.37	0.02	—	—
(II) 1.48	5.5	—	1.33	0.03	—	—
(III) 1.92	6.1	—	1.91	0.03	1.77	—
(IV) 1.07	5.7	0.04	1.09	0.02	0.89	0.05
(V) 1.21	5.9	—	0.97	0.03	0.97	0.07
(VI) 2.70	2.7	0.05	1.03	0.04	1.00	0.06
(VI) 2.70	7.1	0.14	2.72	0.11	2.25	0.14
(VI) 2.70	10.1	0.07	3.19	0.10	2.38	0.06

la protéine à partir de laquelle elles ont été préparées, renferment néanmoins cet acide aminé à un taux parfois voisin de celui caractérisant la thyroglobuline (2.7% au lieu de 3.3%). Toutefois, leur ioduration n'est suivie en aucun cas de la formation de quantités importantes de thyroxine. La signification de ce fait sera discutée plus bas.

3. *Evolution dans le temps de l'ioduration de la caséine.* — Bien avant les travaux de LUDWIG ET VON MUTZENBECHER<sup>1</sup>, de nombreux auteurs, en particulier BLUM ET STRAUSS<sup>17</sup>, BLUM ET VAUBEL<sup>18</sup> avaient établi que les protéines mises en présence d'iode à des  $p_H$  divers s'halogènent rapidement au maximum dans le cycle benzénique de la tyrosine. Par la suite, LUDWIG ET VON MUTZENBERG<sup>1</sup>, REINEKE ET TURNER<sup>3</sup> constataient qu'une incubation prolongée à 37° ou, mieux, à 70° d'iodoprotéines physiologiquement inactives leur confère des propriétés analogues à celles des préparations thyroïdiennes. Il était donc possible qu'une première phase de l'halogénéation conduise spécifiquement à la formation de diiodotyrosine, alors qu'une seconde, beaucoup plus lente, comporterait seule la réaction de condensation donnant naissance à la thyroxine. La plupart des auteurs admettent implicitement ou explicitement cette manière de voir, que nous discuterons dans un prochain mémoire consacré à l'activité biologique des iodoprotéines. Nous nous bornerons à exposer ici les essais que nous avons poursuivis sur la formation de la thyroxine et de ses précurseurs en fonction du temps d'action de l'iode sur la caséine dans des conditions diverses.

Une première série d'expériences a consisté à faire agir à 45° six atomes d'iode en solution (3 g I + 6 g IK + H<sub>2</sub>O q.s.p. 50 ml) par molécule de tyrosine sur de la caséine de Vache en solution à 2% en présence de bicarbonate de sodium à 1.0%. L'iode, ajouté par petites portions, est rapidement fixé par la protéine, la solution ne demeurant que très faiblement colorée en jaune en fin d'expérience. Les temps indiqués dans le compartiment A du Tableau III sont ceux employés pour l'addition de la quantité totale de réactif, l'incubation de la caséine en présence de celui-ci étant interrompue par sa précipitation isoélectrique<sup>2</sup>. Dans une seconde série d'essais, nous avons appliqué la méthode de REINEKE, WILLIAMSON ET TURNER<sup>10</sup> à la préparation d'iodocaséine, mais en opérant à diverses températures et en séparant les produits formés aux temps successifs de la technique décrite par ces auteurs. Enfin, l'ioduration en présence d'oxydes de manganèse renforçant l'activité physiologique des produits, il nous a paru utile de la

TABLEAU III

TENEURS EN IODE TOTAL, EN TYROSINE, EN MONIODOTYROSINE, EN DIIODOTYROSINE ET EN THYROXINE DE CASÉINES IODÉES PAR ACTION DE 6 ATOMES I PAR MOLÉCULE DE TYROSINE DANS DES CONDITIONS DIVERSES

Temps d'action de I et conditions de l'expérience	I total %	Tyrosine %	Monoiodo-tyrosine %	Diiodo-tyrosine %	Thyroxine %
A. IODURATION PAR I EN SOLUTION EN MILIEU BICARBONATÉ À 45°; TEMPS D'IODURATION VARIABLES					
o (témoin)	—	7.20	—	—	—
Essai 1					
5 minutes	7.89	0.40	2.74	6.36	1.14
15 "	9.00	0.34	2.30	6.41	1.87
30 "	9.27	0.36	1.30	6.60	1.66
60 "	9.84	0.28	1.08	6.90	1.77
Essai 2					
3 minutes	8.28	0.32	1.62	7.80	1.06
11 "	10.47	0.38	1.50	7.60	1.21
25 "	10.84	0.31	1.32	7.90	1.53
40 "	11.29	0.27	1.36	6.90	1.62
120 "	10.27	0.22	1.06	6.80	1.83
Essai 3					
5 minutes	8.70	0.43	1.45	6.40	1.10
10 "	8.63	0.33	1.80	6.55	1.22
25 "	9.10	0.39	1.33	6.70	1.37
60 "	8.90	0.33	1.04	6.70	1.70
120 "	10.48	néant	0.85	6.80	1.81
B. IODURATION PAR I EN POUDRE SELON REINEKE, WILLIAMSON ET TURNER; TEMPS ET MODALITÉS D'ACTION DE L'HALOGENE VARIABLES					
2 heures à 37°	9.20	0.22	1.10	6.40	1.21
6 " " 37°	9.30	0.45	1.20	6.30	1.62
6 " " 70°	8.12	0.27	0.80	6.41	1.60
6 " " 70° + Mn <sub>3</sub> O <sub>4</sub>	8.30	0.22	0.90	6.46	1.30
2 h à 37° et 20 h à 70° (technique orig. des auteurs)	9.23	0.16	1.00	7.25	1.33

mettre en œuvre dans une expérience. On trouvera dans le Tableau III les résultats de nos recherches dans ce domaine\*.

La formation de la thyroxine est donc un phénomène très rapide et l'incubation prolongée à diverses températures n'en augmente pas l'intensité de manière appréciable. La signification de ce fait en ce qui concerne le défaut d'activité des iodoprotéines prenant naissance par une halogénéation de courte durée sera envisagée par la suite.

#### DISCUSSION

Deux questions peuvent être utilement discutées à partir des données rassemblées dans les Tableaux I, II et III. Il convient d'envisager, d'une part dans quelle mesure le rendement en thyroxine de l'ioduration des protéines est fonction de leur teneur en tyrosine et, d'autre part, si l'augmentation de l'activité thyroïdienne se manifestant

\* Comme nous l'avons discuté dans un travail antérieur<sup>18</sup>, la méthode colorimétrique de dosage de la thyroxine que nous employons, plus spécifique que diverses autres, donne des résultats moins élevés qu'elles dans les iodoprotéines. C'est la raison pour laquelle les rendements en thyroxine figurant dans ce tableau sont, en apparence, inférieurs à ceux indiqués par REINEKE et ses collaborateurs<sup>3, 10</sup>.

lors de l'incubation des iodoprotéines dans diverses conditions va de pair avec une augmentation de leur teneur en thyroxine.

a. *Teneur en tyrosine, structure des protéines et rendement maximum en thyroxine de l'ioduration.* — Nous avons réuni dans le Tableau IV un ensemble de données établies antérieurement<sup>2, 4</sup> et au cours de ce travail, afin de simplifier la discussion de ces dernières. Les teneurs en thyroxine indiquées sont celles de produits préparés par la méthode de REINEKE et ses collaborateurs<sup>10</sup>, sauf dans les cas de la fibroïne et de la zéïne. Ces deux protéines ont été iodées dans des milieux particuliers (voir paragraphe précédent) en raison de leur insolubilité dans l'eau<sup>4</sup>. Il est néanmoins probable que les résultats obtenus peuvent tous être légitimement comparés, car l'ioduration de la caséine dans les conditions les plus diverses nous a fourni des dérivés à peu près également riches en thyroxine, à condition qu'elle soit opérée par la quantité optima de réactif (6 atomes I par molécule de tyrosine).

TABLEAU IV  
RENDEMENT EN THYROXINE DE L'IODURATION DE PROTÉINES ET DE PEPTONES

Produit étudié	Teneur initiale en tyrosine % (A)	Teneur en thyroxine % après ioduration (B)	Rapport: $\frac{B \times 100}{A}$
Caséine	7.20	1.65	22.9
Fibroïne	12.00	0.40	3.3
Insuline	12.20	1.35	12.7
Thyroglobuline	3.30	0.68	20.6
Zéïne	5.84	1.61	27.5
Peptone IV	1.07	0.04	3.7
Peptone VI	2.70	0.14	5.2

On ne saurait, après examen de ces données, admettre que la teneur en tyrosine d'une protéine régit son aptitude à donner naissance à de la thyroxine. Le premier acide aminé étant la substance-mère du second, son taux est nécessairement important, mais sa position dans la molécule des protéines ou de leurs dérivés paraît être le facteur prédominant du rendement en thyroxine. Celui-ci, dont la dernière colonne du Tableau IV indique les valeurs relatives rapportées à la tyrosine initialement présente, est environ deux fois plus faible dans l'insuline que dans la thyroglobuline et dans la caséine, et la protéine thyroïdienne ne présente à cet égard aucune propriété particulière. Ce dernier fait est en désaccord avec des résultats récemment publiés par RIVIÈRE, GAUTRON ET THÉLY<sup>19</sup> et que nous n'avons pas pu reproduire<sup>20</sup>. Par ailleurs, les peptones de caséine étudiées donnent naissance à des traces de thyroxine par rapport à la tyrosine qu'elles renferment, alors que cette réaction affecte une partie importante de l'acide aminé dans la protéine.

Ces diverses observations relèvent d'une même interprétation, à savoir, que seuls certains restes de tyrosine peuvent, en raison de leur position dans les molécules protéiques, se condenser en thyroxine après transformation en diiodotyrosine. La répartition spatiale de ces restes, leur proximité, sont nécessairement fonction de la structure des diverses protéines et l'action de la pepsine peut, soit les libérer, soit dissocier des molécules dans lesquelles ils occupent une position privilégiée. De toute manière, la totalité des restes de tyrosine ne présente pas dans les protéines une réactivité uniforme

et l'on peut espérer mettre à profit les résultats de l'ioduration pour orienter des recherches sur la répartition et le mode de combinaison de la tyrosine dans les protéines de divers types.

*b. Formation de la thyroxine en fonction du temps d'incubation des protéines dans le milieu d'ioduration et activité biologique.* — De nombreuses observations, dues entre autres à LUDWIG ET VON MUTZENBECHER<sup>2</sup>, à REINEKE, WILLIAMSON ET TURNER<sup>10</sup> ont établi que l'incubation prolongée des iodoprotéines au sein du milieu où elles se forment renforce, parfois même fait apparaître, leur activité biologique. Or, comme il est actuellement certain que la thyroxine se forme à partir de la diiodotyrosine prenant naissance dans un premier temps, REINEKE ET TURNER<sup>21</sup> ont admis que le rendement en la première est très fortement augmenté par la prolongation du temps de chauffage des préparations en solution iodée, surtout en présence d'oxydes de manganèse. Cette hypothèse apparaissait comme d'autant plus plausible que l'incubation à 37° de la diiodotyrosine pure en milieu faiblement alcalin conduit à la formation de thyroxine (VON MUTZENBECHER<sup>22</sup>, JOHNSON ET TEWKESBURY<sup>23</sup>, HARINGTON ET PITT RIVERS<sup>24</sup>). Les données que nous avons établies sur des iodocaséines ayant subi l'action de l'halogène pendant des temps divers (Tableau III) ne sont pas favorables à cette manière de voir.

En effet, le rendement maximum en thyroxine, à 45° et en milieu très faiblement alcalin (CO<sub>3</sub>NaH 1%), est en général atteint en 30 à 60 minutes, la quantité de cet acide aminé présente étant déjà élevée quelques minutes après addition du réactif au taux de six atomes d'iode par molécule de tyrosine. Dans ces conditions, la prolongation du temps de chauffage n'exerce aucune action sur la formation de thyroxine. Il en est de même lorsque l'halogénéation est opérée selon la technique de REINEKE, WILLIAMSON ET TURNER<sup>10</sup>. Dans nos essais, aucune augmentation de la teneur en thyroxine n'a été observée, ni en portant de 37° à 70° la température de chauffage pendant six heures, ni lors de l'addition de Mn<sub>3</sub>O<sub>4</sub> et la technique originale (chauffage de 2 heures à 37°, puis de 20 heures à 70°) conduit à un résultat voisin de celui obtenu à la fin de son premier temps. Or, il n'est pas douteux que les produits préparés par ces diverses techniques sont d'activité biologique très inégale, ceux ayant subi une longue incubation à 70° étant seuls doués à cet égard d'une efficacité importante. Il en découle que celle-ci est liée à des facteurs multiples, dont l'assimilation de la thyroxine présente dans les iodoprotéines est peut-être l'un des plus importants, comme nous le discuterons dans un prochain travail\*. Le fait que REINEKE et ses collaborateurs auraient observé une augmentation de la teneur en thyroxine des iodoprotéines après incubation à 70°, surtout en présence d'oxydes de manganèse, n'est pas en contradiction avec nos résultats, car la méthode de dosage de l'hormone employée par ces auteurs, moins spécifique que la notre, comporte une erreur par excès due sans doute à l'entraînement de produits de dégradation de la thyroxine et de la diiodotyrosine présents dans les iodoprotéines, récemment étudiés par PITT RIVERS<sup>25</sup> (diiodohydroxybenzaldéhyde et triiodophénol). De toute manière, la formation de la thyroxine dans les protéines doit être considérée comme une réaction s'opérant très rapidement en milieu faiblement alcalin; l'activité biologique des produits obtenus paraît dès lors plus liée à l'assimilation de la thyroxine qu'ils renferment qu'au taux de celle-ci.

\* Signalons que TURNER ET REINEKE<sup>26</sup> ont observé que l'utilisation digestive de la thyroxine contenue dans les iodoprotéines est très faible chez les ruminants; elle ne paraît être que de 5% chez le Mouton.

## RÉSUMÉ

1. L'action de l'iode en quantité optima (6 atomes I par molécule de tyrosine) sur les protéines conduit à la formation de thyroxine, de diiodotyrosine et de monoiodotyrosine en proportions diverses selon la nature du produit étudié. Une partie seulement de la tyrosine est susceptible de se transformer en thyroxine dans la caséine, l'insuline, la thyroglobuline, la zéine; l'autre, toujours beaucoup plus importante, demeure à l'état de diiodotyrosine en présence d'un excès de réactif. Le rendement maximum en thyroxine par unité de poids de tyrosine est différent pour chaque protéine. L'interprétation la plus simple de ces faits est que certains restes de tyrosine, seuls aptes à se condenser en thyroxine, occupent dans les protéines une position privilégiée leur conférant une réactivité particulière. L'aptitude d'une protéine à donner naissance à de la thyroxine n'est donc pas seulement fonction de sa teneur en tyrosine, mais aussi de sa structure.

2. Des peptones pepsiques de caséine ne renferment après action de l'iode que des traces de thyroxine, la quasi-totalité de la tyrosine étant alors transformée en dérivé diiodé. Leur comportement vis-à-vis de l'halogène peut tenir soit à la mise en liberté par l'hydrolyse enzymatique des molécules de tyrosine susceptibles de se condenser, soit à la dislocation de la structure les maintenant dans une position favorable.

3. La formation de la thyroxine par ioduration des protéines est une réaction beaucoup plus rapide que ne permet de le prévoir l'évolution de l'activité biologique des préparations en fonction de leur temps d'incubation en présence des réactifs. Elle donne, dans les conditions indiquées, naissance en quelques minutes à des quantités importantes de cet acide aminé.

## SUMMARY

The action of the optimal quantities of iodine (6 atoms iodine per molecule of tyrosine) on proteins results on the formation of thyroxin, diiodotyrosine and monoiodotyrosine in different proportions depending on the protein investigated. Only a part of the tyrosine can be transformed into thyroxin in casein, insulin, thyroglobulin and zein; the main part of the tyroxin remains as diiodotyrosine in the presence of an excess of reagent. The maximum yield of thyroxin per unit of weight of tyrosine is different for each protein. The simplest explanation of these facts is that certain tyrosine residues able to condense to form thyroxin, occupy certain positions in the proteins which make them particularly reactive. Hence the ability of a protein to produce thyroxin is not only dependent on its tyrosine content, but also on its structure.

2. The pepsic peptones of casein, after being treated with iodine, contain only traces of thyroxin, practically all the tyrosine being transformed into the diiodo-derivative. Behaviour of these peptones towards halogens may be due to the liberation by enzymatic hydrolysis of certain molecules of tyrosine or to the break-down of the structure which maintains them in a favourable position.

3. The formation of thyroxin by iodination of proteins proceeds much more rapidly than would be expected from the evolution of the biological activity of the preparations as a function of their incubation time with the reagent. Under the conditions indicated above, considerable amounts of thyroxin are formed in a few minutes.

## ZUSAMMENFASSUNG

1. Die Wirkung von Jod in optimaler Menge (6 Atome I per Mol. Tyrosin) auf Proteine führt zur Bildung von Thyroxin, Dijodtyrosin und Monojodtyrosin in wechselnden Mengen, je nach der Art des untersuchten Proteins. Im Casein, Insulin, Thyroglobulin oder Zein ist nur ein Teil des vorhandenen Tyrosins befähigt, sich in Thyroxin umzuwandeln; der stets viel grössere Teil bleibt als Dijodtyrosin in Gegenwart eines Überschusses an Reagens. Die maximale Ausbeute an Thyroxin per Tyrosin-Gewichtseinheit ist für jedes Protein verschieden. Die einfachste Erklärung dieser Tatsachen ist die, dass gewisse Tyrosinreste, die allein befähigt sind, sich zu Thyroxin zu kondensieren, im Protein eine bevorzugte Stellung einnehmen, die ihnen eine besondere Reaktionsfähigkeit verleiht. Die Fähigkeit eines Proteins, Thyroxin zu bilden, hängt also nicht allein von seinem Tyrosin-gehalt ab, sondern ebenfalls von seiner Struktur.

2. Pepsinpeptone aus Casein enthalten, nach Einwirkung von Jod, nur Spuren von Thyroxin; fast das ganze Tyrosin liegt dann in Form des Dijodderivates vor. Ihr Verhalten gegenüber dem Halogen kann entweder davon herrühren, dass durch die enzymatische Hydrolyse die zur Kondensation befähigten Tyrosinmolekeln abgespalten worden sind, oder davon, dass die Struktur, die sie in günstiger Lage zusammenhielt, auseinandergezerrt worden ist.

3. Die Bildung des Thyroxins durch Jodierung der Proteine ist eine viel raschere Reaktion als die Evolution der biologischen Wirksamkeit der Präparate, bezogen auf ihre Inkubationszeit in Gegenwart der Reagentien, es vorauszusehen erlaubt. Unter den angegebenen Bedingungen werden in wenigen Minuten bedeutende Mengen dieser Aminosäure gebildet.

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# TENEUR EN THYROXINE ET ACTIVITÉ BIOLOGIQUE DE DIVERSES PROTÉINES ARTIFICIELLEMENT IODÉES (CASÉINE, INSULINE, THYROGLOBULINE) ET DE LA THYROGLOBULINE

par

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## I. INTRODUCTION

Des protéines artificiellement iodées d'égale teneur en halogène présentent, selon leur mode de préparation, une activité plus ou moins intense sur la métamorphose des Batraciens, les échanges respiratoires, la créatinurie, la sécrétion lactée et le rythme cardiaque. Cette notion, souvent discutée dans des travaux anciens<sup>1</sup>, a été soumise à un nouvel examen<sup>2, 3, 4, 5</sup>, après que LUDWIG ET VON MUTZENBECHER<sup>6</sup> eurent démontré la formation de thyroxine par ioduration des protéines. Or, s'il est certain que les effets physiologiques de l'administration des iodoprotéines sont dus à cet acide aminé, un problème demeure posé en ce qui concerne les conditions de sa formation et de son efficacité dans les molécules protéiques.

Pour REINEKE ET TURNER<sup>7</sup>, REINEKE, WILLIAMSON ET TURNER<sup>8</sup>, il existe un parallélisme étroit entre l'intensité de l'activité biologique des iodoprotéines et leur teneur en thyroxine, dosée par une méthode établie par REINEKE, TURNER, HOOVER, BEEZLEY ET KOHLER<sup>8</sup> et dérivant de celle de LELAND ET FOSTER<sup>9</sup>. Les techniques de préparation des iodoprotéines actives conduiraient seules à la formation de quantités importantes de cet acide aminé. Pour PITT-RIVERS ET RANDALL<sup>5</sup>, pour DEANESLY ET PARKES<sup>10</sup>, au contraire, l'iode acido-insoluble, lequel correspond à l'iode thyroxinien dans les thyroglobulines, présente dans les iodoprotéines un taux sans relation avec les effets physiologiques de celles-ci. Or, ROCHE ET MICHEL<sup>11</sup>, étudiant le dosage des acides aminés iodés dans les protéines artificiellement halogénées, ont montré que les méthodes de dosage employées dans ces divers travaux comportent une erreur par excès plus ou moins grande et proposé une technique plus spécifique. La mise en œuvre de celle-ci dans des conditions diverses<sup>12</sup> a permis de constater que la formation de thyroxine n'exige pas l'incubation prolongée des iodoprotéines en présence d'iode, opération qui fait apparaître ou renforce leur activité<sup>2, 3</sup>. Aussi devait-on se demander dans quelle mesure les propriétés physiologiques de ces produits ne sont fonction que de leur teneur en thyroxine.

REINEKE ET TURNER<sup>13</sup> ont observé que l'action métabolique d'iodocaséines ingérées par des ruminants correspond à celle d'une très faible fraction de la thyroxine présente (5% chez le Mouton), en sorte que l'assimilation de ces produits paraît commander l'efficacité de leur ingestion. Ainsi s'expliquerait que l'administration à des rats de

quantités importantes d'iodoprotéines n'augmente pas leur métabolisme respiratoire, alors que celle de petites quantités de l'hydrolysât enzymatique de celles-ci est active à cet égard<sup>14</sup>. BRANDT, MATTIS ET NOLTE<sup>15</sup> semblent avoir les premiers formulé cette réserve, que l'on peut faire reposer sur le fondement théorique suivant. La réaction d'oxydation allant de pair avec celle d'halogénéation modifie la structure de la protéine (ouverture de cycles, désamination, déméthylation). Elle doit, en conséquence modifier leur sensibilité aux protéinases digestives ou tissulaires, lesquelles ne libèreraient plus alors la thyroxine qu'avec une extrême lenteur. Le degré d'oxydation des protéines, variable selon les conditions de l'ioduration, paraît donc susceptible d'exercer une action importante sur l'utilisation de la thyroxine présente dans celles-ci. Il en découle que la relation entre l'activité biologique des iodoprotéines et leur teneur en thyroxine n'est pas encore entièrement définie et qu'il y a intérêt à préciser si, comme le pensent REINEKE et ses collaborateurs, la synthèse de la thyroxine est régie par les facteurs qui commandent aussi la formation de produits biologiquement actifs. L'étude chimique de cette réaction pourrait alors tirer profit des données établies dans le domaine biologique.

En dehors de son aspect général, ce problème doit être envisagé sur un autre plan. Le rendement en thyroxine des protéines soumises à l'ioduration, calculé à partir de données établies par voie chimique, n'est pas seulement fonction de leur teneur en tyrosine, mais aussi de leur structure<sup>12</sup>. Il y avait lieu de rechercher en outre si l'activité biologique de diverses protéines halogénées dans des conditions identiques est proportionnelle à leur teneur en thyroxine. Le cas de la thyroglobuline méritait à cet égard d'être particulièrement étudié, car cette protéine pouvait, en raison de sa fonction physiologique, présenter des propriétés spécifiques.

Au demeurant, une question est depuis longtemps posée au sujet de l'activité de la thyroglobuline. BARNES<sup>16</sup>, LERMAN ET SALTER<sup>17</sup>, MEANS, LERMAN ET SALTER<sup>18</sup> ont constaté que l'effet de la thyroxine sur les échanges respiratoires est plus fort lorsque cet acide aminé est présent dans la thyroglobuline que lorsqu'il est libre. Il y avait lieu de confirmer ce fait en ce qui concerne le pouvoir antigoïtrogène des produits thyroïdiens, afin de préciser si l'activité des protéines iodées doit être rapportée directement à leur teneur en thyroxine, comme le font REINEKE ET TURNER. Cette question, à l'étude de laquelle nous espérons apporter une contribution, est importante à résoudre, en ce sens que l'on ne pourra définir l'activité absolue de la thyroglobuline, en l'exprimant par rapport à la thyroxine, que dans la mesure où une réponse lui sera donnée.

Le but de ce travail est de rechercher: 1. dans quelle mesure la voie d'administration, orale ou intrapéritonéale, et la digestion préalable des iodoprotéines modifie leur activité biologique; 2. si la caséine, l'insuline et la thyroglobuline iodées par une même technique sont actives proportionnellement à leur teneur en thyroxine et si leur mode de préparation modifie au même degré cette dernière et leur efficacité physiologique; 3. si l'activité biologique de l'unité de poids de thyroxine est identique dans les préparations de thyroglobuline et dans celles de l'acide aminé pur.

## II. PARTIE EXPÉRIMENTALE

### A. Techniques

Nous avons préparé une série d'échantillons d'iodoprotéines par des techniques diverses et nous en avons étudié l'activité sur la métamorphose de têtards de Batraciens et sur le goitre expérimental du Rat préalablement traité au 6-N propylthiouracile.\*

Trois types d'essais ont été réalisés au cours de ce travail; ils ont comporté respectivement l'ingestion d'iodoprotéines, celle de leurs hydrolysats tryptiques et l'injection intrapéritonéale des premières.

Les procédés d'ioduration employés sont, soit l'halogénéation en milieu ammoniacal par l'iode d'azote<sup>2, 19</sup>, laquelle donne naissance à des produits inactifs ou peu actifs, que nous désignerons au cours de ce travail sous le nom d'iodoprotéines A, soit l'ioduration à  $p_H = 7.5-8.0$  en solution renfermant du bicarbonate de sodium à un taux voisin de 1%. Cette opération a été réalisée dans des conditions diverses. Pour REINEKE et ses collaborateurs, l'addition d'iode en poudre à des solutions de caséine en milieu bicarbonaté de  $p_H = 7.8$ , opérée par petites portions en 2 heures à  $37^\circ$ , conduirait à la fixation de l'halogène sans conférer à la protéine d'activité importante et sans provoquer une synthèse notable de thyroxine. Cette dernière évoluait par contre en 20 heures, lorsque le milieu est, dans un second temps, chauffé à  $70^\circ$  sous agitation continue. Aussi avons-nous préparé des iodocaséines soit par la méthode originale des auteurs américains appliquée dans ses deux temps (produits B), soit en arrêtant les opérations à la fin du premier (produits C). Enfin, nous avons réalisé dans un certain nombre de cas l'ioduration en milieu bicarbonaté par l'iode en solution agissant à  $45^\circ$  pendant 30 minutes<sup>12</sup>, les produits D obtenus étant inactifs sur les échanges respiratoires, bien que relativement riches en thyroxine. Dans tous les cas choisis comme exemples, nous avons fait réagir 6 atomes I par molécule de tyrosine, le rendement le plus élevé en thyroxine étant alors obtenu<sup>3, 7, 12, 16</sup>.

Des caséines, des insulines et des thyroglobulines pures ont été iodées par ces diverses techniques. La caséine (Vache) a été préparée par la méthode de LINDERSTRØM-LANG<sup>20</sup>, la thyroglobuline par celle de DERRIEN, MICHEL ET ROCHE<sup>21</sup>; l'insuline était un produit cristallisé de diverses origines, dont les échantillons employés renfermaient tous 12.2% de tyrosine\*. Les dérivés iodés de ces protéines ont été analysés, leur teneur en iode total étant déterminée par la méthode de LEIPERT<sup>29</sup> et leur teneur en thyroxine par les méthodes de REINEKE, TURNER, KOHLER, HOOVER ET BEEZLEY<sup>8</sup> et de ROCHE ET MICHEL<sup>11</sup>. Cette dernière donne seule des résultats ne comportant probablement pas d'erreur par excès; il a néanmoins paru utile d'opérer dans un certain nombre de cas le dosage décrit par REINEKE et ses collaborateurs, afin de tenir compte de ses résultats dans la discussion. On trouvera dans le Tableau I les valeurs obtenues, auxquelles nous avons joint des données relatives à des préparations de thyroglobuline, les unes pures, les autres brutes (extrait thyroïdien total).

L'activité de ces produits sur la métamorphose du têtard de *Bufo bufo* et sur le poids du corps thyroïde de rats porteurs d'un goitre expérimental au 6.N-propylthiouracile a été étudiée. Les essais poursuivis sur Batracien ont constitué une recherche d'orientation, car le manque de spécificité de la réponse des têtards à de multiples composés iodés empêche de lui attribuer une valeur absolue. Néanmoins, la valeur relative des informations que l'on peut recueillir par son étude quantitative dans des conditions soigneusement standardisées ne saurait être mise en doute. Nous avons poursuivi cette étude par la méthode de DEANESLY, EMMETT ET PARKES<sup>23</sup>. Pour cela, des lots de cinq têtards de *Bufo bufo* mesurant de 28 à 32 mm de long, immergés dans 250 ml d'eau

\* Nous remercions très vivement les Professeurs J. C. DRUMMOND (Nottingham), E. JORPES (Stockholm) et K. LINDERSTRØM-LANG (Copenhague) et les firmes BOOTS, VITRUM ET LEO, de nous avoir adressé les échantillons d'insuline cristallisée utilisée pour l'ensemble de nos travaux sur les iodoprotéines.

TABLEAU I  
TENEURS EN IODE TOTAL ET EN THYROXINE DE DIVERSES IODOPROTÉINES

No. et nature de la protéine étudiée	I %	Thyroxine % dosée selon	
		ROCHE ET MICHEL	REINEKE et collaborateurs
Caséine iodée A No. 1	10.40	0.85	—
Caséine iodée A No. 1bis	10.20	0.83	1.70
Caséine iodée B No. 2	8.80	1.27	2.20
Caséine iodée B No. 12	9.23	1.23	2.90
Caséine iodée C No. 8	8.80	1.25	—
Caséine iodée C No. 9	7.98	0.86	—
Caséine iodée C No. 12bis	8.60	1.33	2.70
Caséine iodée D No. 17	8.40	1.25	—
Insuline iodée C	14.80	1.35	—
Thyroglobuline pure iodée C*	3.65	0.65	—
Thyroglobuline pure (I'orc)*	0.41	0.21	—
Thyroglobuline brute (I'orc)	0.38	0.21	—

\* Valeurs moyennes de trois préparations de compositions très voisines

TABLEAU II  
ACTION DU 6.N-PROPYLTHIOURACILE SUR LE POIDS ET LA TENEUR EN IODE DU CORPS THYROÏDE DE RATS MÂLES DE 60 À 80 g PLACÉS À 22° PENDANT 10 JOURS

Nombre d'animaux mis en expérience	Quantité de 6.N-propylthiouracile administrée par jour et par 100 g de poids corporel	Poids du corps thyroïde frais (mg par 100 g de poids corporel)	Iode total thyroïdien (mg par 100 g d'organe frais)
15 (témoins)	0	17.5	57
3	0.02	21.5	45
3	0.05	19.0	28
3	0.18	27.9	16
3	0.50	60.6	6
3	0.50	53.0	—
3	1.17	58.3	2
12	2.00	57.8	traces
9	2.35	55.7	"
3	4.90	57.0	"
3	7.50	74.5	"
3	20.00	60.2	"

ont reçu pendant un jour une dose définie d'iodoprotéine ajoutée à l'eau dans laquelle ils baignent. Les lots d'animaux ayant ingéré totalement le produit sont ensuite placés pendant deux jours dans la même quantité d'eau à 25° et l'on mesure leur taille en fin d'expérience. Les lots de têtards ayant incomplètement absorbé les produits offerts sont éliminés. Les essais ont été en général poursuivis sur quatre doses différentes de chaque protéine et se sont montrés reproductibles.

L'action antigotrogène de la thyroxine a été démontrée par DEMPSEY ET ASTWOOD<sup>24</sup> et mise à profit par REINEKE, MIXNER ET TURNER<sup>25</sup> pour établir un test biologique précis d'activité sur lequel il a été possible de baser le dosage de celle-ci. Nous inspirant de leurs travaux et de ceux de FRIEDEN ET WINZLER<sup>26</sup>, nous avons mis en œuvre comme agent gotrogène le 6.N-propylthiouracile. La dose quotidienne minima de ce produit faisant disparaître l'iode du corps thyroïde et provoquant une hypertrophie importante de cet organe en dix jours a tout d'abord été déterminée. On trouvera dans le Tableau II

les résultats d'expériences que nous avons poursuivies dans ce but sur des rats mâles d'un même élevage, pesant de 60 à 80 grammes, placés dans une pièce climatisée à 22° et ingérant la substance goitrogène mélangée à leur régime alimentaire (ratigène fourni par *Alimentation équilibrée*, Commentry).

Ces résultats ont permis de choisir l'administration quotidienne pendant 10 jours de 2 mg de 6.N-propylthiouracile comme la condition la plus favorable à la constitution d'un goitre important sans risquer d'intoxication due à l'excès de la substance active\*. Les animaux ainsi traités ont en outre reçu pendant 10 jours des doses croissantes des produits dont nous cherchions à définir l'activité ou des substances de référence: DL-thyroxine, thyroglobuline pure ou brute (extrait thyroïdien). On a déterminé le poids frais de leur corps thyroïde en fin d'expérience et celui d'animaux normaux mis au régime de base dans les mêmes conditions. Chaque dose a été administrée à trois ou six rats et la comparaison des poids des organes provenant d'animaux traités a été faite avec le poids moyen du corps thyroïde de douze témoins n'ayant reçu que l'agent goitrogène pendant 10 jours et avec celui de douze animaux normaux ingérant le régime de base pur. La différence  $\Delta$  entre le gain de poids par 100 g de poids corporel de la glande des rats recevant l'agent goitrogène seul ou en présence d'un produit iodé (par rapport aux rats normaux) a été calculée. Ce mode opératoire nous a donné des résultats très satisfaisants, à condition d'employer des lots homogènes d'animaux et de maintenir constante à 22° la température des locaux où sont placés ceux-ci. Il y a lieu, néanmoins, de ne considérer comme rigoureuses les comparaisons qu'il permet de faire, que si elles sont opérées avec des séries témoins établies pour chaque essai. L'action antigoitrogène, mise en œuvre selon la technique dont la justification vient d'être présentée, nous a permis d'étudier l'efficacité comparée de produits administrés par voie digestive ou intrapéritonéale et celle de leurs hydrolysats tryptiques, alors que les dosages biologiques basés sur la métamorphose des Batraciens ne sont faciles à réaliser avec des têtards de petite taille que par administration orale de la substance<sup>5</sup> à étudier.

Celle-ci a été soit ingérée, soit injectée par voie intrapéritonéale. Dans le premier cas, elle a été mélangée à une certaine quantité de régime dont on a contrôlé l'utilisation quantitative par les animaux. Dans le second, elle a été mise en solution à  $p_H = 7.5-7.8$  et injectée sous un volume ne dépassant pas 2 ml. Les hydrolysats enzymatiques d'iodocaséines ont été préparés par action de la trypsine à  $p_H = 7.8$  et à 37° pendant 12 heures environ, près de 40% de l'azote total de ces produits étant alors libéré à l'état aminé, comme nous avons pu nous assurer par le dosage de N aminé par la méthode de VAN SLYKE\*\*.

### B. Résultats expérimentaux

Notre étude a porté sur quinze cents têtards et cinq cent vingt rats et ses résultats ne sauraient être présentés que sous forme abrégée. Les essais sur Batracien seront très brièvement résumés, car ils ont surtout servi à orienter ceux basés sur l'action antigoitrogène des dérivés iodés chez le Rat. Les uns et les autres ne sont comparables avec précision que s'ils ont été poursuivis avec un même lot homogène d'animaux et à peu près simultanément; tel est toujours le cas pour ceux que nous avons réunis dans une

\* La teneur en iode du corps thyroïde des animaux ayant reçu pendant 10 jours une dose quotidienne de 2 mg de 6.N-propylthiouracile tend à redevenir normale après 6 à 8 jours de régime non additionné d'agent goitrogène. C'est pourquoi nous avons administré simultanément celui-ci et ses antagonistes.

\*\* L'incubation à 37° dans les mêmes conditions, mais en l'absence de trypsine, ne modifie pas l'activité des iodocaséines; l'augmentation de celle-ci décrite plus bas après hydrolyse tryptique des produits B est donc bien due à la protéolyse.

même figure. En ce qui concerne les essais sur le Rat, un lot d'animaux témoins a été adjoint à chaque série d'expériences, car la réponse du corps thyroïde au 6.N-propylthiouracile et aux produits iodés présente des différences non négligeables d'une saison à l'autre. Par exemple, l'ingestion quotidienne de 2 mg du premier pendant 10 jours a provoqué chez nos animaux une hypertrophie thyroïdienne par 100 g de poids corporel plus forte (de 14.2 à 62.0 mg en moyenne) en octobre qu'en mai (de 17.5 à 57.0 mg en moyenne). De plus, le pouvoir antigéotrogène des iodoprotéines naturelles ou artificielles s'est montré sensiblement plus élevé au printemps qu'à l'automne. Ces faits expliquent

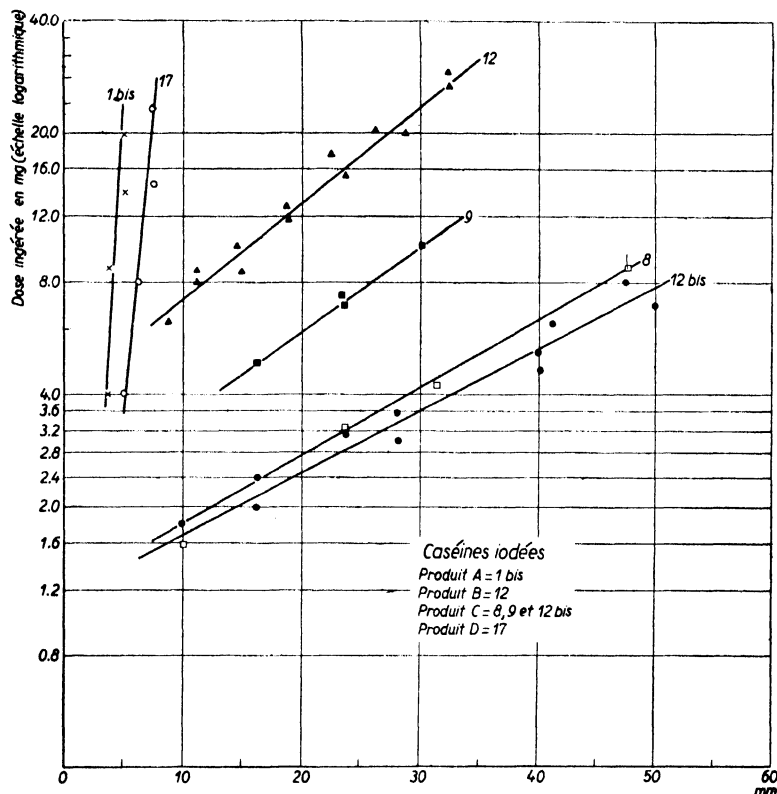


Fig. 1. Activité de caséines iodées préparées par des techniques diverses (voir Tableau I) sur la métamorphose du têtard de *Bufo bufo*. Abscisses: diminution de la longueur totale en millimètres (lot de 5 animaux). Ordonnées: logarithme de la dose d'iodoprotéine administrée, exprimée en milligrammes ingérés par lot de 5 têtards

pourquoi les courbes traduisant l'activité des produits étudiés présentent des différences d'une figure à l'autre pour un même corps. Ces différences sont parfois notables dans les valeurs des témoins (Fig. 9); néanmoins, elles sont sans conséquence sur l'exactitude comparée des résultats déterminés sur un seul lot d'animaux.

Les Figs 1, 2 et 3 ont été établies à partir d'exemples choisis parmi ceux illustrant les résultats des quatre-vingts expériences poursuivies. Leur mode de présentation, traduisant le raccourcissement corporel en fonction du logarithme de la dose de produit administrée, objective l'efficacité de celui-ci et la régularité de son action.

Fig. 2. Activité de la thyroglobuline pure de Porc (A), de l'iodoprotéine en dérivant (B) et d'une caséine iodée choisie comme produit de référence (C) sur la métamorphose du têtard de *Bufo bufo*

Abscisses: diminution de la longueur totale en millimètres (lot de 5 animaux). Ordonnées: logarithme de la dose d'iodoprotéine administrée, exprimée en milligrammes ingérés par lot de 5 têtards

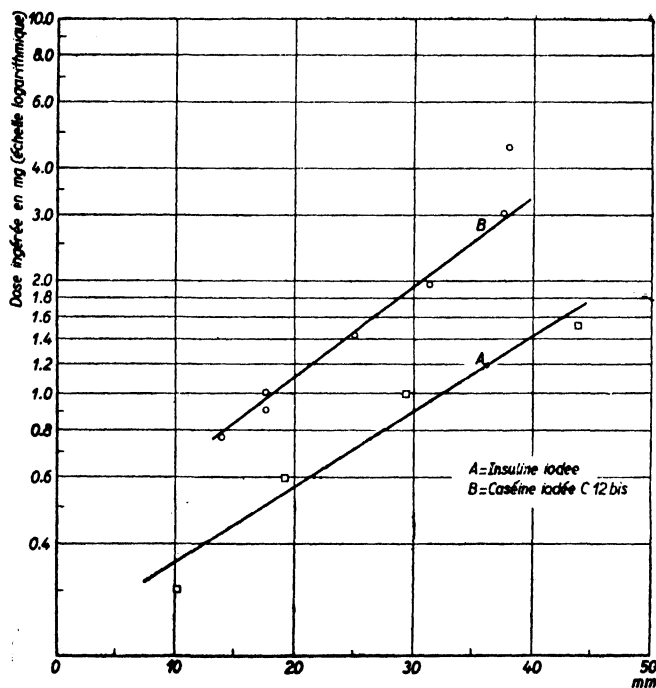
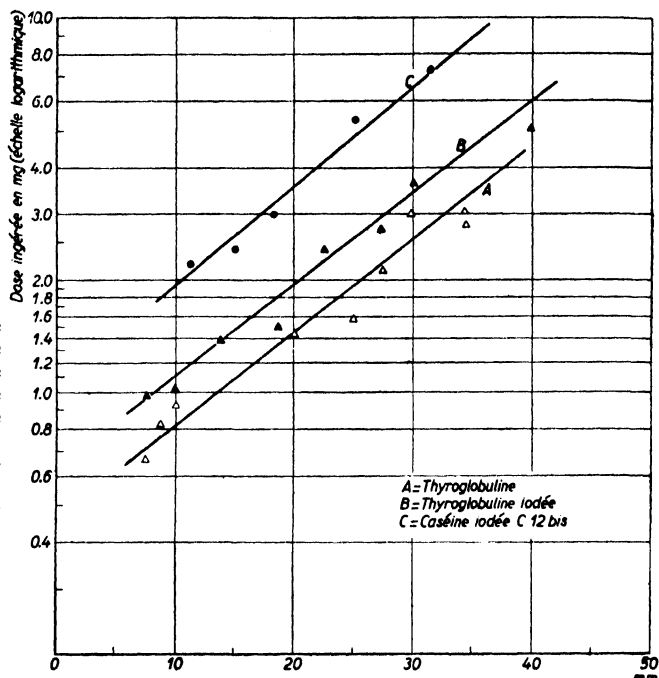


Fig. 3. Activité de l'insuline iodée (A) et d'une caséine iodée choisie comme produit de référence (B) sur la métamorphose du têtard de *Bufo bufo*

Abscisses: diminution de la longueur totale en millimètres (lot de 5 animaux). Ordonnées: logarithme de la dose d'iodoprotéine administrée, exprimée en milligrammes ingérés par lot de 5 têtards

L'examen de la Fig. 1 fait ressortir l'inégalité d'action des iodocaséines selon leur mode de préparation. Les produits A (ioduration en milieu ammoniacal) et D (ioduration rapide en milieu bicarbonaté par I en solution) n'accélèrent pratiquement pas la métamorphose du têtard de *Bufo bufo*, bien que renfermant de la thyroxine. Par ailleurs, le produit B (ioduration par le premier temps de la méthode de REINEKE et collaborateurs) est, à égalité de teneur en celle-ci, beaucoup moins actif que les produits C, et dans le cadre de ceux-ci l'efficacité des préparations 8, 9 et 12 bis, étudiées s'ordonne selon leur richesse en thyroxine. La Fig. 2 illustre les résultats obtenus avec la thyroglobuline

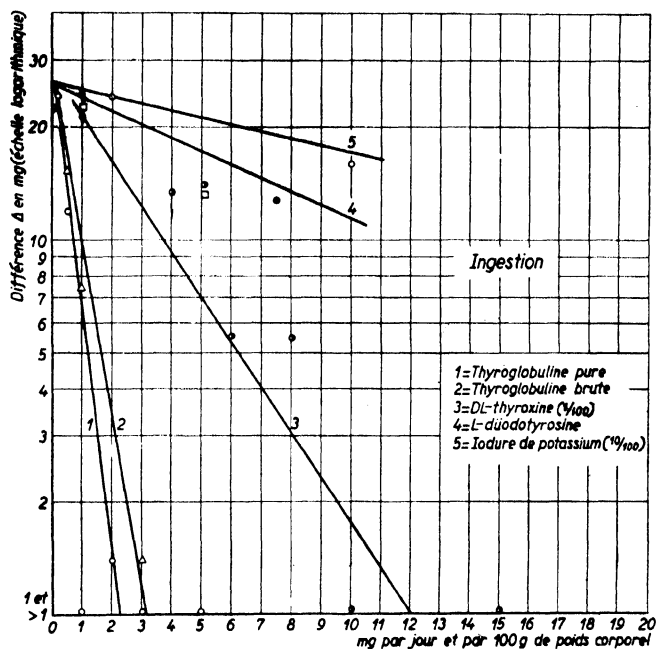


Fig. 4. Activité antigotrogène (6.N-propylthiouracile) de thyroglobuline brute et pure (Porc) de L-diiodotyrosine, de DL-thyroxine et d'iodure de potassium. Abscisses: mg par jour et par 100 g de poids corporel ingérés (protéines administrées en substances, L-diiodotyrosine pure, DL-thyroxine diluée à 1% dans la caséine et iodure de potassium dilué à 10% dans cette dernière). Ordonnées: Δ, différence (mg) du gain de poids du corps thyroïde, en 10 jours d'expérience, chez les témoins goitreux et chez les animaux traités\* (échelle logarithmique)

naturelle et avec son produit d'ioduration et permet de les comparer avec ceux donnés par l'étude d'une caséine choisie comme produit de référence. La caséine iodée C 12 bis a été retenue en cette qualité, comme étant la plus active de celles que nous ayons préparées sur une large échelle.

Plusieurs faits sont objectivés par cette figure. D'une part la thyroglobuline est, à poids égal, plus active que la caséine iodée C 12 bis, bien qu'environ six fois moins riche en thyroxine. D'autre part l'enrichissement de la thyroglobuline en celle-ci par ioduration n'augmente pas son activité; il paraît au contraire la diminuer. Enfin, la Fig. 3 établit que l'insuline iodée est, à égalité de teneur en thyroxine, à peu près deux fois plus active sur la métamorphose du têtard de *Bufo bufo* que la caséine iodée C 12 bis.

\* Gain de poids de l'organe p 100 g de poids corporel, calculé à partir de données de référence établies sur des animaux témoins normaux de même poids corporel.



Fig. 5. Activité antigoitrogène (6.N-propylthiouracile) de caséines iodées par diverses techniques

Abscisses: mg par jour et par 100 g de poids corporel ingérés. Ordonnées:  $\Delta$ , différence (mg) du gain de poids du corps thyroïde, en 10 jours d'expérience, chez témoins goitreux et chez les animaux traités (échelle logarithmique)

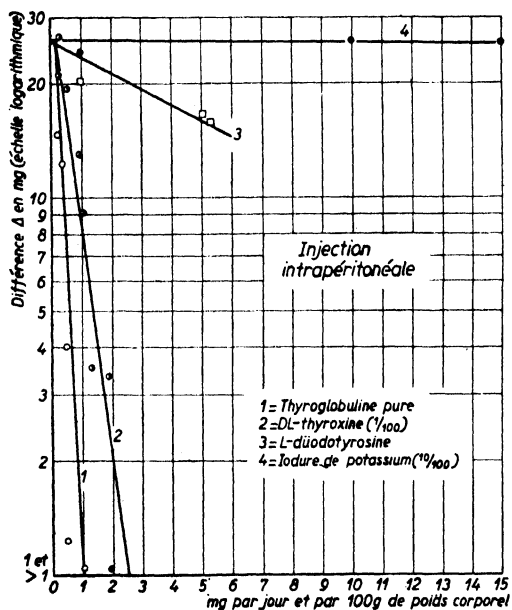
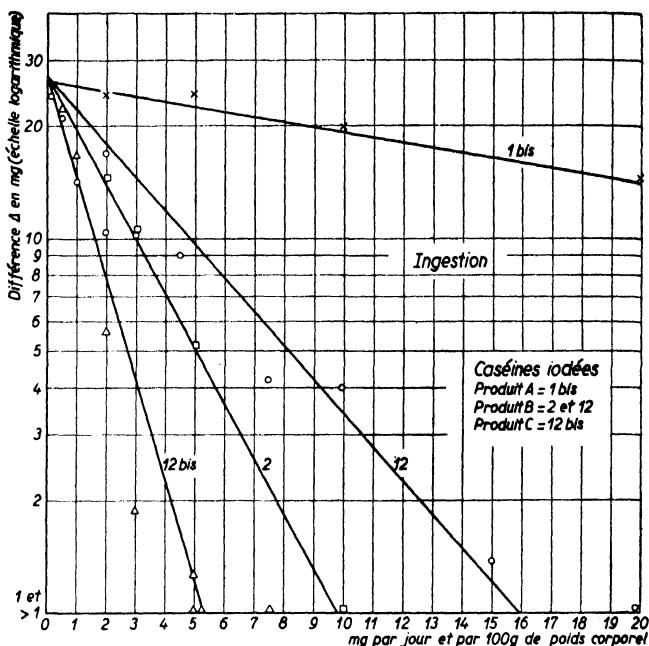


Fig. 6. Activité antigoitrogène (6.N-propylthiouracile) de la thyroglobuline pure (Porc); de la DL-thyroxine, de la L-diiodotyrosine et de l'iodure de potassium administrés par injection intrapéritonéale. Abscisses: mg par jour et par 100 g de poids corporel ingérés (thyroglobuline administrée en substance, L-diiodotyrosine pure, DL-thyroxine diluée à 1% dans la caséine et iodure de potassium dilué à 10% dans cette dernière. Ordonnées:  $\Delta$ , différence (mg) du gain de poids du corps thyroïde en 10 jours d'expérience chez les témoins goitreux et chez les animaux traités (échelle logarithmique)

Les Figs 4, 5, 6, 7, 8 et 9 rapportent un ensemble de résultats obtenus dans l'étude de l'action antigoitrogène (6.N-propylthiouracile) d'iodoprotéines et de dérivés iodés divers administrés par voie buccale ou parentérale, certaines iodoprotéines ayant subi au préalable une hydrolyse partielle sous l'action de la trypsine. Un mode de présentation uniforme a été adopté dans ces figures. La réduction du gain de poids du corps thyroïde

par rapport à la glande des témoins goitreux a servi de test de l'efficacité des produits iodés. Il convenait de la rapporter objectivement à la quantité de ceux-ci administrés dans les conditions précisées plus haut. Après divers essais, nous avons choisi comme système de coordonnées, en abscisses le nombre de milligrammes de produit iodé administré par jour et par 100 grammes de poids corporel et, en ordonnées, la différence  $\Delta$ , du gain de poids du corps thyroïde après 10 jours d'expérience chez les témoins goitreux recevant uniquement du 6.N-propylthiouracile, et chez les animaux traités en outre par des produits iodés le poids de l'organe étant dans tous les cas rapporté à 100 g de poids corporel.  $\Delta$  a été exprimé en milligrammes et reporté sur une échelle logarithmique. Le gain du poids de l'organe a été calculé par la différence: poids moyen du corps thyroïde des animaux normaux témoins — poids moyen de l'organe des animaux recevant à la dose indiquée soit l'antigoitrogène seul, soit celui-ci et un produit iodé. Les Figs 4 et 5 résument les données obtenues lors de l'ingestion d'iodoprotéines et de produits de référence divers. Les thyroglobulines, l'iodoinsuline et les iodocaséines ont été administrées à des doses ne dépassant pas 20 mg par jour et par 100 g de poids corporel, la diiodotyrosine à celle de 1 à 10 mg. La DL-thyroxine\* a été ingérée à l'état de dilution au centième dans de la caséine et l'iodure de potassium mélangé à 90% de cette protéine. Il en découle que les abscisses des Figs 4 et 5 correspondent à l'ingestion quotidienne de mg de produits, les uns purs (thyroglobulines, iodocaséines, diiodotyrosine), les autres (thyroxine, iodure de potassium), dilués dans un excipient inactif.

Les faits observés dans cette première série d'essais corroborent ceux que l'étude des mêmes produits sur la métamorphose du têtard de *Bufo bufo* avait établis. L'action antigotrogène de la caséine 1 bis (produit A, iodé en milieu ammoniacal) est presque nulle, celle de la caséine 12 bis (produit C, iodé par la méthode de REINEKE et collaborateurs) étant plus élevée que celle des préparations 2 et 12, halogénées par application du premier temps (chauffage de 2 heures à 37°) de la même méthode. La thyroglobuline est, à égalité de poids, plus active que la caséine C 12 bis, puisque 2 à 3 mg par jour de la première empêchent le développement du goitre expérimental en 10 jours, alors que 5 à 6 mg de la seconde, pourtant six fois plus riche en thyroxine, sont nécessaires pour atteindre le même résultat. L'iodure de potassium et la diiodotyrosine sont à peu près inefficaces. Quant à l'action de la DL-thyroxine ingérée, elle présente des irrégularités depuis longtemps connues, mais est néanmoins plus faible, pour cet acide aminé libre que pour le même poids de ce corps compris dans la thyroglobuline. En effet, 12 mg d'une dilution au centième de thyroxine dans la caséine, soit 0.12 mg de l'hormone (DL) font disparaître en 10 jours le goitre expérimental, alors que 0.004 à 0.006 mg de celle-ci (soit 2 à 3 mg de thyroglobuline) conduisent au même résultat. La signification de ce fait sera discutée plus bas.

Les Figs 6, 7 et 8 résument les résultats obtenus lors de l'injection intrapéritonéale des mêmes produits dissous en milieu neutre ou très faiblement alcalin ( $p_H = 7, 6-7, 8$ ) et d'iodoinsuline.

L'iode injecté à l'état d'iodure est inactif, la diiodotyrosine très peu active; les iodocaséines le sont à des degrés divers, la thyroglobuline et l'iodoinsuline intensément, comme en témoigne l'examen de la Fig. 6. La thyroglobuline est beaucoup plus efficace que la thyroxine, puisque 2.5 mg d'une dilution à 1% de l'hormone (DL), soit 0.025 mg, ont la même action antigotrogène que 1 à 2 mg de la protéine, c'est-à-dire à 0.004 mg

\* Nous remercions la maison HOFFMANN-LA ROCHE (Bâle) d'avoir mis à notre disposition la thyroxine pure utilisée dans ces recherches.

de thyroxine au maximum. L'action de cette dernière est, comme il est depuis longtemps établi, beaucoup plus régulière par voie parentérale que par voie digestive.

Parmi les iodocaséines, les produits A (halogénéation en milieu ammoniacal) demeurent les moins actifs; les effets des produits B (halogénéation selon le premier temps de la méthode de REINEKE et ses collaborateurs) sont inégaux pour les caséines 2 et 12. Il est remarquable que l'injection de celle-ci soit équivalente à celle de la caséine C 12 préparée par la méthode de REINEKE et ses collaborateurs, alors que son ingestion est beaucoup moins efficace. Enfin, le pouvoir antigotrogène de l'iodoinsuline est environ

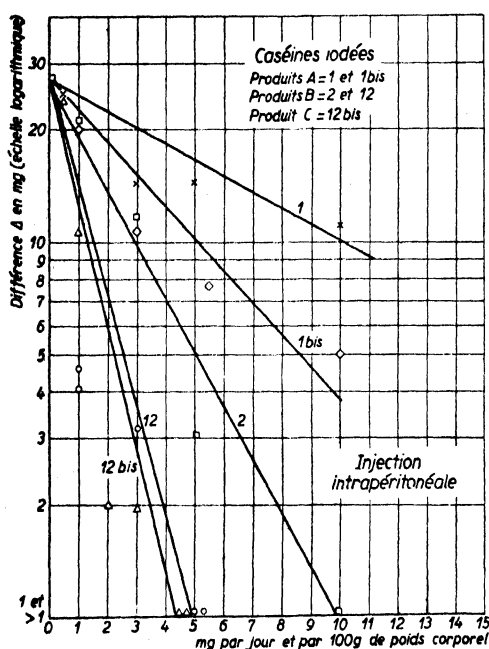


Fig. 7. Activité antigotrogène (6.N-propylthiouracile) de caséines iodées par diverses techniques, administrées par injection intrapéritonéale. Abscisses: mg par jour et par 100 g de poids corporel injectés. Ordonnées:  $\Delta$ , différence (mg) du gain de poids du corps thyroïde, en 10 jours d'expérience, chez les témoins goitreux et chez les animaux traités (échelle logarithmique)

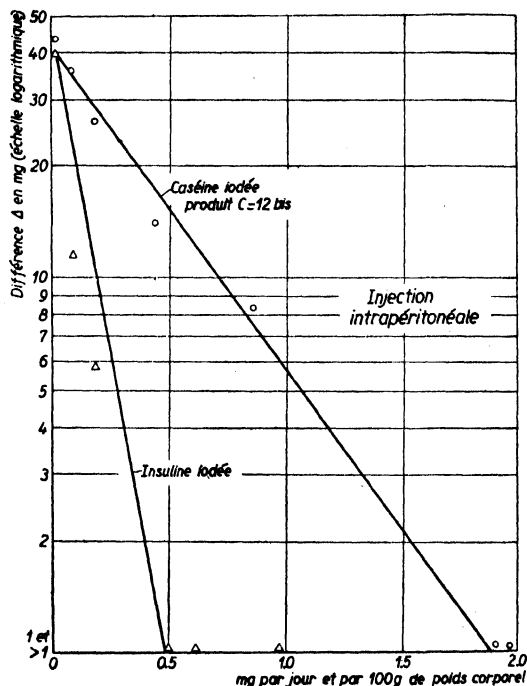


Fig. 8. Activité antigotrogène (6.N-propylthiouracile) de l'insuline iodée et d'une caséine iodée (C 12 bis) prise comme produit de référence, administrées par injection intrapéritonéale. Abscisses: mg par jour et par 100 g de poids corporel injectés. Ordonnées:  $\Delta$ , différence (mg) du gain du poids du corps thyroïde en 10 jours d'expérience, chez les témoins goitreux et chez les animaux traités (échelle logarithmique)

quatre fois plus forte que celui de l'iodocaséine C 12 bis, également riche en thyroxine; il est, à poids égal, supérieur à celui de la thyroglobuline. Néanmoins, cette dernière demeure nettement la plus active par unité de poids d'hormone, puisque 0.007 mg de thyroxine présente dans l'iodoinsuline équivalent au plus à 0.004 mg du même corps dans la thyroglobuline (valeurs approchées).

Ces observations ont orienté nos recherches vers le rôle de l'utilisation digestive des iodoprotéines comme facteur de leur efficacité; aussi y avait-il intérêt à étayer cette notion sur les résultats d'expériences plus précises, à partir desquels a été établie la

Fig. 9. Un premier essai a été poursuivi sur l'action de la même préparation de thyroglobuline pure administrée par ingestion et par injection intrapéritonéale. Par ailleurs, comme les caséines B 12 et C 12 bis, inégalement actives par voie buccale, présentent une activité voisine par voie parentérale, une expérience devait être entreprise pour démontrer que leur comportement dans le premier cas est dû à leur utilisation digestive incomplète. Nous l'avons réalisée en soumettant ces deux caséines à une hydrolyse trypsique *in vitro* avant de la faire ingérer avec le 6.N-propylthiouracile. On trouvera rassemblées dans la Fig. 9 les courbes traduisant l'activité de la thyroglobuline ingérée

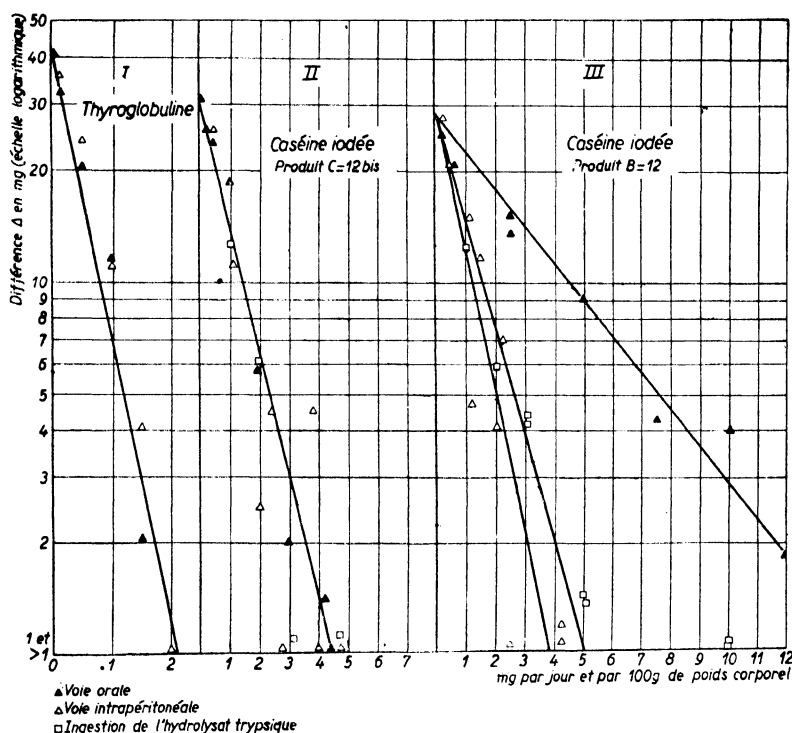


Fig. 9. Activité antigotrogène (6.N-propylthiouracile): I, de la thyroglobuline de Porc administrée par voie orale et par injection intrapéritonéale; II, de la caséine C 12 bis ingérée sans traitement préalable ou après digestion trypsique et injectée; III, de la caséine B 12 ingérée sans traitement préalable ou après digestion trypsique et injectée. Abscisses: mg par jour et par 100 g de poids corporel injectés. Ordonnées: Δ, différence (mg) du gain de poids du corps thyroïde chez les témoins goitreux et chez les témoins traités (échelle logarithmique)

ou injectée et celle des caséines B 12 et C 12 bis ingérées avant ou après hydrolyse trypsique et injectées.

Ces essais, poursuivis dans des conditions particulièrement précises, puisqu'ils ont comporté l'administration de nombreuses doses de produits actifs dans une marge de quantité assez étroite, ont donné des résultats significatifs. D'une part, ils ont montré que la thyroglobuline est aussi bien utilisée par voie orale que par voie parentérale chez le Rat. D'autre part, la caséine C 12 bis, identiquement efficace par injection ou par ingestion et qu'elle soit ou non hydrolysée au préalable par la trypsine, présente une activité que son assimilation digestive ne paraît pas limiter. Il n'en est pas de même,

de celle de la caséine B 12, puisque ce produit, relativement peu efficace par voie buccale s'il n'a subi aucune digestion trypsique, le devient beaucoup plus après celle-ci; il est alors presque aussi actif qu'injecté par voie intrapéritonéale. La résorption commande donc, dans une certaine mesure, le pouvoir antigotrogène des iodoprotéines.

### III. DISCUSSION

Le commentaire des figures ci-dessus a rendu compte des faits observés, dont il convient maintenant de dégager la signification. Celle-ci peut-être discutée sur les plans biochimique et physiologique, après avoir défini dans quelle mesure les résultats obtenus dans l'étude des deux propriétés biologiques mises en œuvre peuvent être coordonnés.

L'action des produits iodés les plus divers sur la métamorphose des Batraciens ne permet d'attribuer au test basé sur ce phénomène qu'un intérêt limité. Des discordances entre les résultats obtenus dans son étude et dans celle des échanges gazeux d'animaux recevant les mêmes produits ont été fréquemment signalées; aussi n'est-il pas surprenant qu'il en existe entre eux et ceux de l'action antigotrogène sur le plan quantitatif. Par exemple, la thyroglobuline est à poids égal environ deux fois plus active sur la métamorphose du têtard que la caséine C 12 bis, alors qu'elle l'est sensiblement plus sur le poids du corps thyroïde des rats. De même, l'iodoinsuline est deux fois plus active que la caséine C 12 bis dans le premier test, quatre fois plus dans le second. Il en est sans doute ainsi parce que d'autres produits iodés que la thyroxine présents dans les protéines halogénées exercent une action indirecte sur la métamorphose, en tant que source d'iode pour la synthèse de thyroxine réalisée par le têtard. Il demeure par ailleurs possible que les Amphibiens soient doués d'une susceptibilité particulière à certains dérivés thyroïdiens résultant de l'hydrolyse des iodoprotéines<sup>27</sup>. En outre, la détermination de la dose quotidienne de produit actif empêchant totalement la formation du goitre, objectivée par la présentation des résultats que nous avons adoptée, permet une mesure quantitative de l'activité des corps étudiés. Celle-ci comporte une marge d'imprécision due au fait que la réponse des animaux et le comportement des témoins varie sensiblement d'une série à l'autre d'essais, mais les comparaisons que l'on peut opérer dans le cadre de chacune de celles-ci sont significatives. Pour ces diverses raisons, nous avons attribué aux données établies sur la métamorphose du têtard de *Bufo bufo* un caractère d'orientation, et nous avons surtout retenu pour la discussion de ce travail les résultats exprimant l'action antigotrogène des corps étudiés.

La mesure la plus précise que l'on puisse donner de celle-ci est de la rapporter à la dose quotidienne (mg p. 100 g de poids corporel) minima inhibant l'action gotrogène 2 mg de 6.N-propylthiouracile ingérés quotidiennement pendant 10 jours. La quantité ainsi déterminée n'est pas rigoureusement constante d'une expérience à l'autre, mais son ordre de grandeur le demeure, en sorte que l'on peut baser sur elle une approximation satisfaisante. On trouvera dans le Tableau III un ensemble de données significatives à ce sujet, établies en admettant avec PITT RIVERS ET LERMANN<sup>28</sup>, que la L-thyroxine est 10 fois plus active que l'isomère D, du même corps.

La discussion des résultats peut être abordée à partir des faits que traduit l'examen des Tableaux I et III et des Figs 1 à 9.

a. *Teneur en thyroxine et activité biologique des protéines artificiellement iodées.* On ne saurait mettre en doute le fait que la méthode de préparation des iodoprotéines commande leur efficacité physiologique, mais le déterminisme des faits observés doit être

TABLEAU III  
ACTIVITÉ ANTIGOITROGÈNE RELATIVE DE DIVERS PRODUITS RAPPORTÉE A CELLE DE LA  
L-THYROXINE (MOYENNES)

Produit et voie d'administration	Quantité minima antigoitrogène (mg/j/100 g) (A)	Quantité de L-thyroxine pré- sente dans (A) (B)	Activité de (B) rappor- tée à la quantité minima de L-thyroxine douée du même pouvoir anti- goitrogène (inject.) (C)
DL-thyroxine (orale) . . . . .	0.12	—	0.18
(intrapéritonéale) . . . . .	0.02	—*	1.00
Thyroglobuline (orale) . . . . .	2.0	0.004	2.75
(intrapéritonéale) . . . . .	2.0	0.004	2.75
Iodoinsuline (intrapéritonéale) . .	0.5	0.007	1.57
Caséine iodée B 12 (orale) . . . .	16.0	p.191	0.06
(intrapéritonéale) . . . . .	4.0	0.049	0.22
Caséine iodée 12bis (orale) . . . .	4.0	0.043	0.25
(intrapéritonéale) . . . . .	4.0	0.043	0.25

\* Activité correspondant à 0.01 mg de L-thyroxine administrée avec le racémique et à 0.01 mg de dérivé D, soit à 0.001 mg de l'isomère L. Valeur de (B) pour 0.02 mg de DL-thyroxine = 0.011 mg de dérivé L, choisie comme unité dans la colonne (C). Cette définition de la valeur de (B) pour la DL-thyroxine est imposée par le fait que l'activité du racémique est comparée dans la colonne (C) à celle de la thyroxine des iodoprotéines, qui est l'isomère L (REINEKE ET TURNER) dans le cas des produits obtenus par action de l'iode en milieu neutre.

précisé. Pour REINEKE, TURNER, KOHLER, HOOVER ET BEEZLEY<sup>8</sup>, il existe une concordance satisfaisante entre l'activité métabolique (augmentation des échanges respiratoires) des iodoprotéines et leur teneur en L-thyroxine, déterminée par voie biologique (augmentation des échanges respiratoires après injection intrapéritonéale de l'hormone ou par la méthode chimique qu'ils ont proposée). Nos résultats ne sont pas entièrement favorables à cette manière de voir, puisque certains produits riches en thyroxine sont d'une activité nulle ou minime et que, par ailleurs, leur digestibilité est très inégale.

L'efficacité d'une iodoprotéine injectée par voie intrapéritonéale est nécessairement liée à son hydrolyse, sans laquelle la thyroxine ou des peptides diffusibles de celle-ci ne peuvent entrer dans le sang et les humeurs. Or, nous avons constaté que diverses iodocaséines de même teneur en thyroxine, également actives après digestion trypsique, ne le sont pas quand on les ingère sans qu'elles aient subi cette opération (Fig. 9). Il est donc logique d'admettre que les protéases humorales ou cellulaires permettant aux tissus d'assimiler les iodoprotéines injectées se comportent vis-à-vis de celles-ci comme les enzymes du tractus digestif, en sorte que *l'activité d'une iodoprotéine est commandée non seulement par sa teneur en thyroxine, mais par la vitesse de libération enzymatique de l'hormone*. Ainsi, des produits riches en thyroxine, mais résistants à l'hydrolyse digestive, peuvent-ils être rejetés avant que l'acide aminé iodé ait été assimilé et, s'ils sont injectés, la libération de celui-ci ne s'opère qu'avec une lenteur telle que son action est répartie sur un temps trop long pour qu'elle apparaisse intense.

Il est possible que REINEKE et ses collaborateurs aient obtenu des produits dont l'activité réponde aux normes indiquées par eux, mais celles-ci traduisent l'utilisation intégrale de la thyroxine présente dans les iodoprotéines injectées et il est peu probable que ce fait doive être observé dans tous les cas, même dans les conditions les plus favorables. La méthode adoptée par REINEKE ET TURNER pour préparer des iodopro-

téines actives est à coup sûr la plus satisfaisante et le second des temps qu'elle comporte augmente très sensiblement l'activité des produits obtenus administrés par voie orale; mais il n'en est ainsi qu'en raison de l'augmentation de leur degré d'assimilation, non de leur enrichissement en thyroxine. L'étude de l'iodoinsuline préparée par la même méthode apporte un autre argument défavorable à l'opinion dont nous discutons le bien-fondé. A égalité de teneur en thyroxine, la caséine C 12 *bis*, halogénée selon la méthode des auteurs américains, est, en effet, environ huit fois moins active en injection intrapéritonéale\*. Un facteur important autre que la teneur en thyroxine doit donc participer au déterminisme des effets physiologiques et pharmacologiques des iodo-protéines. La sensibilité de ces dérivés à l'hydrolyse enzymatique doit être mise en cause à la suite de nos essais. Elle explique que deux produits puissent être inégalement actifs, bien que renfermant une même quantité de thyroxine. L'étude de l'activité de la thyroxine et de la thyroglobuline a seule permis de discuter sous ses divers aspects le problème étudié.

*b. Activité relative et activité absolue des protéines iodées et de la thyroglobuline.* Rapporter l'activité biologique de la thyroxine à son unité de poids et la considérer comme une constante physiologique implique l'acceptation d'un postulat: à savoir que les effets de ce produit se manifestent avant qu'il ait été métabolisé. Or, ABELIN ET WEHREN<sup>29</sup> ont montré que son injection est suivie rapidement de sa fixation partielle dans le foie, fait confirmé par l'étude de la destinée de l'hormone marquée par de l'iode radioactif en position 3' et 5'<sup>30</sup>. Un important travail d'ensemble sur la fixation en fonction du temps de la thyroxine marquée, poursuivi par GROSS ET LEBLOND<sup>31</sup>, a établi que chez le Rat, environ 50% de ce produit injecté (0.8 mg) par voie intraveineuse sont fixés et excrétés en deux heures par le foie et le tube digestif, le premier opérant alors la désioduration de 25% de l'hormone avec libération d'ions I<sup>-</sup>. Par ailleurs, HARINGTON<sup>32</sup>, passant récemment en revue les résultats des recherches consacrées à l'action pharmacodynamique de la thyroxine, en particulier sur des organes isolés, attache une grande importance au fait qu'elle n'est pas instantanée, mais comporte un temps perdu assez long pour que l'on doive se demander si l'hormone n'est pas au préalable métabolisée par un processus lent. Dès lors, il apparaît très difficile de définir l'activité *absolue* de la thyroxine et de lui rapporter celle d'une iodoprotéine.

Ces réserves devaient être formulées avant de discuter les résultats obtenus sur la thyroglobuline. Comme l'avaient observé sur le plan qualitatif divers auteurs<sup>16, 17, 18</sup>, l'unité de poids de thyroxine présente dans cette protéine, également efficace quelle que soit sa voie d'administration (voir Fig. 9), est beaucoup plus active que l'acide aminé libre. Ce dernier, en partie détruit dans l'intestin quand il est ingéré, exerce alors une action antigotrogène irrégulière (voir Fig. 4), tandis que son injection va de pair avec une diminution de poids du corps thyroïde hypertrophié assez constante pour une même dose, dans une même série d'essais. Une interprétation englobant ces divers faits peut être proposée. Puisque la thyroxine injectée est partiellement détruite, on peut admettre que sa dégradation s'opère régulièrement dans des conditions expérimentales identiques; de ce fait son activité biologique par unité de poids est peu variable, mais ne correspond en réalité qu'à celle d'une fraction du produit administré. Par ailleurs, l'injection de l'hormone provoque une augmentation très forte et très brutale de la thyroxinémie et,

\* Les essais sur têtard de *Bufo bufo*, résumés sur la Fig. 2, montrent que l'ioduration de la thyroglobuline en diminue l'activité, bien que l'enrichissant en thyroxine. Nous nous bornons à signaler cette observation, n'ayant pas étudié le pouvoir antigotrogène de la thyroglobuline iodée.

par là même, à la fois une saturation des récepteurs et un ensemble de "fuites" (fixation hépatique, élimination digestive et urinaire). Si, au contraire, la thyroxine ne pénètre dans les humeurs que lentement et à dose très faible dans l'unité de temps, son utilisation sera probablement meilleure et ses pertes par les émonctoires seront minimales. L'hydrolyse enzymatique de la thyroglobuline dans le tractus digestif ou dans la cavité péritonéale et dans le sang conduit à cette pénétration lente de l'hormone dans les humeurs, laquelle est probablement à l'origine des faits que nous discutons; *l'unité de poids de la thyroxine est sans doute plus efficace dans la thyroglobuline qu'à l'état libre parce qu'elle est plus complètement utilisée dans la première.*

L'étude du cas particulier de la thyroxine permet une interprétation générale de nos résultats, en ce sens que l'activité thyroïdienne d'une iodoprotéine naturelle ou artificiellement halogénée est, dans tous les cas, liée à deux facteurs principaux : sa teneur en thyroxine et la vitesse de pénétration de celle-ci dans les humeurs qui la véhiculent aux cellules. Alors que la thyroxine libre injectée à dose physiologiquement efficace est partiellement détruite ou éliminée, celle apportée par la thyroglobuline non dénaturée ou par l'iodoinsuline C que nous avons étudiée, est sans doute mieux utilisée, parce qu'elle pénètre dans les humeurs avec une vitesse permettant aux cellules de la fixer moins incomplètement. Les préparations de caséine iodée préparées en vue de ce travail renferment, par contre, de la thyroxine paraissant moins active que l'acide aminé libre, en raison de son faible coefficient d'utilisation. La valeur de celui-ci domine l'efficacité biologique des iodoprotéines et, par là même, leurs applications thérapeutiques ou zootechniques.

#### RÉSUMÉ

1. L'action de diverses iodoprotéines artificielles (iodocaséine, iodoinsuline, iodothyroglobuline) de la thyroglobuline naturelle et de la thyroxine sur la métamorphose du têtard de *Bufo bufo* et sur le goitre expérimental du Rat au 6.N-propylthiouracile a été étudiée dans des conditions permettant de comparer les effets propres à ces divers produits.

2. Des iodocaséines préparées par diverses méthodes et de teneurs en thyroxine voisines présentent une activité biologique très inégale par unité de poids d'hormone lorsqu'elles sont ingérées; ces différences s'atténuent et peuvent même disparaître lorsque les mêmes protéines sont injectées ou soumises à l'action de la trypsine préalablement à leur ingestion. Il en découle que l'efficacité biologique de ces produits est liée non seulement à leur teneur en thyroxine, mais à l'assimilation de celle-ci. Des travaux antérieurs ont sans doute attribué à une augmentation de la première des phénomènes dus à une amélioration de la seconde.

3. L'iodoinsuline et la thyroglobuline naturelle présentent une activité biologique plus élevée que celle de l'acide aminé libre par unité de poids de thyroxine. La discussion des faits observés permet de conclure qu'il en est ainsi parce que l'hormone contenue dans ces protéines est plus complètement utilisée que la thyroxine libre, en raison de son passage progressif dans les humeurs au cours de la protéolyse.

#### SUMMARY

1. The action of various iodinated proteins (iodocasein, iodoinsulin, iodothyroglobulin), of natural thyroglobulin and of thyroxin on the metamorphosis of tadpoles of *Bufo bufo* and on experimental goitre in rats fed on a diet containing 6.N-propylthiouracil has been studied under strictly comparative conditions.

2. Iodocaseins prepared by various methods and containing nearly the same thyroxin percentage are of very different activities when administered by mouth; these differences are less marked and can even disappear when the same products are injected or when submitted to tryptic hydrolysis. The biological efficiency of the iodinated proteins, thus, depends not only on their thyroxin content, but also on the assimilation of the hormone. Previous researches have given results interpreted as showing differences in thyroxin content of iodoproteins when an increase of assimilation was obtained.

3. Iodoinsulin and natural thyroglobulin show, per unit weight of thyroxin, a biological activity greater than free thyroxin. The data obtained in this work lead to the conclusion that the hormone present in these proteins is better utilized than free thyroxin, owing to its progressive penetration in the body fluids during the proteolysis.



## ZUSAMMENFASSUNG

1. Die Wirksamkeit verschiedener künstlicher Jodproteine (Jodcasein, Jodinsulin, Jodthyroglobulin) und des Thyroxins auf die Metamorphose der Kaulquappe von *Bufo bufo* und auf den experimentellen Kropf der gleichzeitig mit der Nahrung 6.N-Propyl-thiouracil empfangenden Ratte wurde untersucht, unter Bedingungen welche einen Vergleich der Eigenwirkungen dieser verschiedenen Substanzen gestatten.

2. Auf verschiedene Art bereitete und in ihrem Thyroxingehalt ähnliche Jodcaseine zeigen eine sehr ungleichmässige biologische Wirksamkeit per Hormon-Gewichtseinheit wenn sie per Os verabfolgt werden; diese Unterschiede werden schwächer und können sogar verschwinden wenn die gleichen Proteine eingespritzt oder vor ihrer Verabfolgung der Trypsin-Einwirkung ausgesetzt werden. Daraus folgt, dass die biologische Wirksamkeit dieser Produkte nicht nur von ihrem Thyroxingehalt abhängt, sondern dazu noch von der Assimilierung des Hormons. Frühere Untersuchungen haben, zweifellos irrtümlich, Erscheinungen auf einen höheren Thyroxingehalt zurückgeführt, welche auf einer besseren Thyroxin-Assimilierung beruhten.

3. Jodinsulin und natürliches Thyroglobulin zeigen per Thyroxin-Gewichtseinheit eine höhere biologische Wirksamkeit als die freie Aminosäure selbst. Die Betrachtung der beobachteten Tatsachen gestattet den Schluss, dass das in diesen Proteinen enthaltene Hormon besser verwendet wird als freies Thyroxin, wegen seines allmählichen Eindringens in die Körperflüssigkeit im Verlauf der Proteolyse.

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## THE CHEMICAL ASSAY OF BIOLOGICALLY ACTIVE IODINATED PROTEINS: ISOLATION OF THYROXINE

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### INTRODUCTION

According to HARINGTON AND RANDALL<sup>1</sup>, the thyroid gland contains only two iodinated compounds; thyroxine and diiodotyrosine. Thyroxine, which is the active principle of the gland, can be separated from diiodotyrosine by virtue of its insolubility in acid solution. This is the basis of the chemical method of thyroid assay (HARINGTON AND RANDALL<sup>2</sup>) in which thyroid substance is hydrolysed with alkali, the thyroxine fraction of the hydrolysate is precipitated with acid and the iodine of the precipitate determined either directly or indirectly (total iodine minus acid-soluble iodine gives acid-insoluble or thyroxine iodine). The technique of the method has been criticized by several workers mainly on the grounds that hydrolysis is inadequate and that diiodotyrosine iodine is therefore included in the acid-insoluble fraction. Nor is the method always accepted on theoretical grounds: the relationship between biological activity and acid-insoluble iodine was questioned by GADDUM AND HETHERINGTON<sup>3</sup>; these authors believed that the activity of the gland was related to the total rather than the thyroxine iodine. However, TAUROG AND CHAIKOFF's<sup>4</sup> recent evidence in favour of thyroxine being the circulating thyroid hormone indicates that the thyroxine content of any preparation is likely to be the best index of its activity.

HARINGTON AND RANDALL's<sup>2</sup> method of chemical assay cannot be used with biologically active iodinated proteins, first prepared by LUDWIG AND VON MUTZENBECHER<sup>5</sup> in 1939 and since then extensively studied by other workers. During iodination, acid-insoluble iodine-containing products other than thyroxine are formed, so that the acid-insoluble iodine content is no longer a guide to the thyroxine content of the proteins. This was amply demonstrated when a number of iodinated protein preparations were made from casein, "Ardein" (ground-nut protein), and ox-plasma by PITT-RIVERS AND RANDALL<sup>6</sup> for experiments on the milk yield response in cows (BLAXTER<sup>7</sup>). Preliminary chemical assays by the method of HARINGTON AND RANDALL<sup>2</sup> were done in the expectation that products with high acid-insoluble iodine contents would show high physiological activity. The chemical assay however, was no guide to activity, and in later experiments on the effect of iodinated proteins on the metamorphosis of *Xenopus* tadpoles, DEANESLY AND PARKES<sup>8</sup> confirmed these findings, that the acid-insoluble iodine content of many proteins tested (prepared by different workers) could not be correlated with their activity; these authors however found that biological response as measured on tadpoles was quantitatively related to the response in cows. Later PITT-

RIVERS<sup>9</sup> showed that two iodinated proteins with high acid-insoluble iodine values and low activity yielded only minute amounts of thyroxine after alkaline hydrolysis.

Evidence is brought forward from the experiments described below that the activity of artificially iodinated proteins is proportional to their thyroxine content. A simple method for thyroxine isolation is described.

#### EXPERIMENTAL

For these experiments, six iodinated proteins were chosen with different biological activities. One of them (casein C4 + 5) was according to DEANESLY AND PARKES<sup>8</sup> slightly more active than thyroid powder when administered in an equal dose; another (casein NCB 3/62) had an activity very similar to that of thyroid powder. The iodinated proteins used include casein and "Ardein" preparations and one plasma preparation.

#### Hydrolysis and isolation

The iodinated proteins (30–35 g) were boiled under reflux in an electric heating mantle for 20 hours with 200 ml water and 100 g hydrated baryta. (These quantities were all doubled for the hydrolysis of Plasma N<sub>4</sub> as the expected amount of thyroxine was small). The precipitated barium salts were separated and decomposed with 1% NaOH and Na<sub>2</sub>SO<sub>4</sub> according to the method of HARRINGTON<sup>10</sup>. A small crop of acid-insoluble material was also obtained from the baryta filtrate and was combined with the product from the barium salt. After drying, this material was extracted twice with ether and dissolved in the minimum amount of boiling 0.1 N sodium carbonate solution and filtered. The sodium salt of thyroxine was allowed to separate in the ice-chest during 72 hours after which it was collected by centrifuging; the salt was dissolved in

the same volume of boiling 0.1 N sodium carbonate solution and acidified with acetic acid (c.f. TAUROG AND CHAIKOFF<sup>4</sup>). The crystalline thyroxine obtained was collected, washed with water and dried *in vacuo*. Melting points of the samples of thyroxine obtained varied between 228° and 235° (decomp.). One of the iodinated proteins "Ardein" N<sub>4</sub>SF) was also hydrolysed with sodium hydroxide and the thyroxine isolated by n-butanol extraction according to LELAND AND FORSTER<sup>11</sup>. The results of these experiments are summarized in the last column of Table I; other data on the iodinated

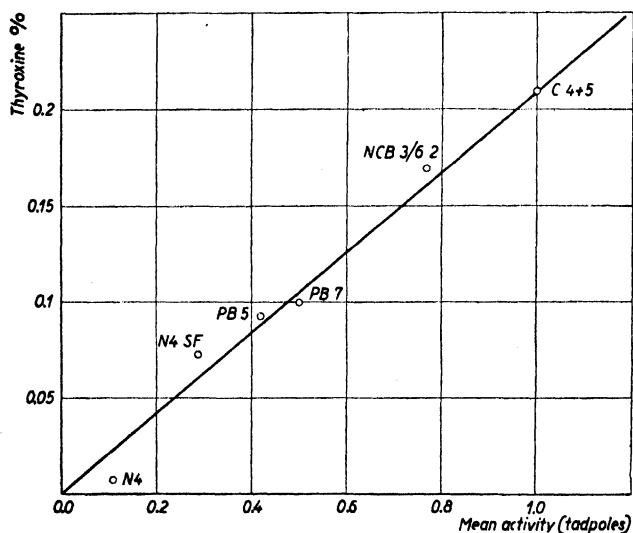


Fig. 1. Relationship between the thyroxine content by isolation and biological activity of iodinated proteins

proteins investigated are taken from DEANESLY AND PARKES's<sup>8</sup> paper. The relationship between the biological activity of iodinated proteins and their thyroxine content as determined by isolation is shown in Fig. 1.

TABLE I

Iodinated Protein	Iodine %		Mean biological activity (tadpoles)	Thyroxine % by isolation
	Total	Acid-insoluble		
Casein C 4 + 5	8.1	1.6	1.0	0.21
Casein NCB 3/62	8.02	1.46	0.77	0.17
"Ardein" PB7(DT/S/824)	7.2	1.2	0.50	0.10
"Ardein" PB5(DT/S/822)	7.3	1.3	0.42	0.094
"Ardein" N4SF	3.61	0.50	0.29	0.074*
				0.071*
Plasma N4	5.4	0.4	0.11	0.007

\* Duplicate isolation by LELAND AND FOSTER's<sup>11</sup> method

## DISCUSSION

The above findings indicate that the biological activity of iodinated proteins is directly proportional to their thyroxine content when they are compared with each other. Of greater interest is the consideration whether the activity of such iodinated proteins can be compared with the activity of thyroid powder, the comparison being based on the thyroxine content of equivalent doses. Now DEANESLY AND PARKES<sup>8</sup> emphasized that the most active iodinated protein tested by them with 1-2% acid-insoluble iodine had only a slightly greater activity than commercial thyroid powder with 0.1% acid-insoluble iodine. On an acid-insoluble iodine basis therefore the artificially iodinated proteins are 10-20 times less active than thyroid powder. If however one considers the amount of thyroxine administered in equal doses of iodinated protein and thyroid powder then the biological responses to these doses can be explained in terms of thyroxine content only. For instance, the iodinated protein investigated in the present work whose activity was most similar to that of thyroid powder was the casein preparation NCB 3/62, and was found to contain 0.17% of thyroxine by isolation; thyroid preparations prepared according to the British Pharmacopoeia are standardized to contain approximately 0.15% thyroxine (0.099 to 0.11% thyroxine iodine). The dose of active principle is of the same order in both cases. It is therefore suggested that the thyroxine content of an artificially iodinated protein determined by isolation will give the most reliable index of its biological activity.

I am indebted to my colleague Dr. A. S. PARKES and to Dr. D. TRAILL, Imperial Chemical Industries (Explosives) Ltd., for gifts of iodinated proteins.

## SUMMARY

1. Crystalline thyroxine has been isolated from six iodinated proteins of known biological activity.
2. The amount of thyroxine isolated was found to be proportional to the biological activity of the proteins.
3. On the basis of thyroxine content, the activity of the iodinated proteins may be compared with that of thyroid powder.

## RÉSUMÉ

1. La thyroxine a été isolée de six protéines iodées dont l'activité biologique est connue.

References p. 678.

2. La quantité de thyroxine isolée est proportionnelle à l'activité biologique des protéines.
3. L'activité biologique de ces protéines peut être comparée à celle de la poudre de thyroïde à l'égard de leur teneur en thyroxine.

#### ZUSAMMENFASSUNG

1. Kristallisiertes Thyroxin wurde aus sechs Jodoproteinen bekannter biologischer Aktivität isoliert.
2. Die isolierte Thyroxinmenge ist der biologischen Aktivität der Proteine proportional.
3. Die Aktivität der Jodoproteine kann auf Grund des Thyroxingehaltes mit derjenigen von Thyroidpulver verglichen werden.

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## SOME NITROGENOUS CONSTITUENTS OF WORT AND THEIR FATE DURING FERMENTATION BY TOP AND BOTTOM FERMENTATION YEASTS\*

by

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### I. INTRODUCTION

The nitrogenous fraction of wort is mainly composed of substances of varying complexity, *e.g.*, ammonia, amides, amino acids, peptides (di- and polypeptides), and proteins. Other nitrogenous bodies, which are unrelated chemically to the proteins are also present such as the bases, choline, betaine and the purine base allantoin, but the part these substances play in yeast metabolism is at present obscure.

In 1929 S. B. SCHRYVER AND E. M. THOMAS<sup>1</sup> made a preliminary examination of the nitrogen groupings present in strong worts (O.G. 1057), for example, ammonia N, amide N, amino N and peptide N. In this way they were able to account for 59 % of the total soluble nitrogen, leaving an undetermined residue of 41 %. In a number of respects, however, the methods of determination were faulty (see below), more especially in the estimation of amide N and polypeptide N.

The data available at the present time about the nature of the individual components of these nitrogen groupings are very meagre and little is known about their fate during fermentation. H. T. BROWN<sup>2</sup> was able to identify the amide asparagine and the amino acids, leucine, tryptophan and tyrosine, as well as the bases choline, betaine and the purine base allantoin in a cold water extract of malt, while O. MÍSKOVSKÝ<sup>3</sup> claimed to have identified the amino acids, arginine and histidine and the bases choline and betaine in Pilsener beer.

In view of the paucity of data on the nature of the various nitrogenous constituents of wort and of their great importance for yeast reproduction, the whole problem has been re-examined. The greater part of this investigation is concerned with top-fermentation yeast and worts prepared by the infusion method; but some data are also included of fermentations carried out with decoction worts and bottom-fermentation yeasts.

### II. METHODS

**Material.** The first part of this investigation deals with the fate of the following nitrogen groupings during fermentation; Total soluble nitrogen, ammonia N, amide N,

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amino N and protein N. All top-fermentations were carried out in the laboratory in Dewar cylinders on pale ale wort (O.G. 1032 and 1040) at the relatively high temperatures of top-fermentation brewing (18° to 22° C). The lager wort (O.G. 1041) was fermented under the normal conditions of a bottom-fermentation system\*.

#### *Methods of Analysis*

- a. *Total Nitrogen* was estimated by the usual Kjeldahl method.
- b. *Total Crystalloid Nitrogen* (non-protein N) was determined on solutions which had been cleared of protein with colloidal ferric hydroxide by the method of W. THOMAS<sup>4</sup>. The cleared extracts were acidified with acetic acid, evaporated to dryness and the nitrogen estimated as for total nitrogen.
- c. *Ammonia Nitrogen* was estimated by the method of G. W. PUCHER, H. B. VICKERY, AND C. S. LEBENWORTH<sup>5</sup>.
- d. *Amide Nitrogen* was determined on the cleared wort by hydrolysis with 5% sulphuric acid for 5 hours and the ammonia present estimated by the PUCHER *et al.* method.
- e. *Amino Nitrogen* was estimated by the method of C. G. POPE AND M. F. STEVENS<sup>6</sup>. It should be mentioned here that too great weight must not be placed on the figures for amino N, because this method includes some dipeptides and polypeptides. The results are, however, more reliable than the VAN SLYKE nitrous acid method. It is hoped to repeat these determinations so as to estimate true  $\alpha$ -amino N in wort by the VAN SLYKE ninhydrin method<sup>7</sup>.
- f. *Amino Acids*. Sixteen amino acids present in wort were estimated quantitatively by microbiological assay (see E. C. BARTON-WRIGHT<sup>8, 9</sup> and E. C. BARTON-WRIGHT AND N. S. CURTIS<sup>10</sup> and their fate followed in fermentation, while two amino acids (glycine and  $\alpha$ -alanine) were also shown to be present in wort by "partition chromatography" (see R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN<sup>11</sup>).

Since in the present investigation we are concerned with fluctuations of definite chemical substances and not merely with amide N or amino N, the results are expressed in the following terms.

*Asparagine N*. The assumption was made that the whole of the amide N is present in wort in the form of amides of the type of asparagine and glutamine. Asparagine N is therefore taken as twice the amide N.

*Amino Acid N*. This was found by deducting amino N due to amide from the total N recorded by the POPE AND STEVENS method.

*Residual N*. This term (see E. G. MASKELL AND T. G. MASON<sup>12</sup> and E. C. BARTON-WRIGHT AND A. McBAIN<sup>13</sup>) is used to cover the fraction of crystalloid N not accounted for by the sum of the asparagine N, amino acid N and ammonia N.

*Protein N*. This value was obtained by deducting total crystalloid N (non-protein N) from total nitrogen.

### III. EXPERIMENTAL OBSERVATIONS

The figures for ammonia N, asparagine N, *etc.*, for a number of fermentations on worts of different gravities are given in Table I.

*Total Soluble N*. The fall in total soluble N follows the usual course with top-fermentation yeasts (Series I, II and III). The greatest uptake of nitrogen occurs during the first 24 hours of fermentation. In the bottom-fermentation (Series IV), the period of greatest nitrogen uptake is later (approximately 48 hours). A result no doubt due to the lower temperature at which fermentation is carried out. The values obtained here for the bottom-fermentation should be compared with the results obtained by E. HELM AND B. TROLLE<sup>14</sup> which they fully confirm.

*Total Crystalloid N*. The fall in total crystalloid N in the main follows the same course as total soluble N.

*Amino Acid N*. It has already been mentioned that the amino acid N figures include some dipeptide and lower polypeptide N owing to the method of estimation used. On the other hand, the POPE AND STEVENS method gives a truer picture of amino acid N

\* We are indebted to Messrs BARCLAY, PERKINS AND Co, Ltd., London, for generous supplies of lager wort at different stages of fermentation.

fluctuations than the VAN SLYKE nitrous acid method, which is known to estimate ammonia N, polypeptide N and other substances such as formaldehyde. A few estimations were made in this investigation by the VAN SLYKE method for comparison purposes, and in every instance the values were 25% higher than with the POPE AND STEVENS method. With this qualification the amino acid N figures given here show that top-fermentation yeasts can absorb up to 62% of the total amino acid N of wort whereas bottom-fermentation yeasts take up less (approximately 42%). In this case the figures given in Table I (Series IV) should be compared with those of Table VI.

*Ammonia N.* There are marked differences in the fate of ammonia N in top and bottom-fermentation systems. In all the cases quoted here top-fermentation yeasts absorb 84–86% of the ammonia N present in wort, whereas initial absorption to the extent of 90% is followed by excretion to the extent of 31% in a bottom-fermentation (Table I, Series IV).

*Asparagine N.* The most outstanding result shown from Table I, and one contrary to expectation, is to be found in the behaviour of asparagine N in both top and bottom-fermentation systems. Following upon H. T. BROWN's discovery (*loc. cit.*) of asparagine in a cold water extract of malt, it was shown by R. S. W. THORNE<sup>15</sup> in a comparative study of ammonia (as ammonium phosphate) and a large number of individual amino acids as single sources of nitrogen supply for yeast growth, that asparagine (or its free acid, aspartic acid) is 42% superior to ammonium phosphate as a nitrogen nutrient, which is itself superior to any of the other amino acids which were tested. The only exception is glutamic acid which is slightly superior to ammonium phosphate. In wort, however, in the presence of ammonia and a mixture of amino acids and peptides, the behaviour of asparagine N is different. Whereas in top and bottom-fermentation systems ammonia N is utilized by yeast to the extent of 84–90%, asparagine N is only taken up to the extent of approximately 30% in the early stages of fermentation and this absorption is followed by excretion; the final concentration of asparagine N being in some cases (Table I, Series III) higher than the initial value. In view of THORNE's findings, this behaviour of asparagine N in wort is unexpected, and in such circumstances, it does not apparently play an important role as a nitrogen nutrient for yeast.

*Residual N.* The residual N figure for worts prepared by the infusion method varies between 41 and 48% while a figure of 41% was found for the single example examined of the decoction process (Table I, Series IV). The nature of the constituents of this fraction have not yet been determined. SCHRYVER AND THOMAS<sup>1</sup> gave a residual N figure of 41% for wort. They claimed to have estimated polypeptide N, excluding polypeptide N, the figure of 41% has been obtained in two cases in this investigation. It is doubtful if SCHRYVER AND THOMAS actually estimated true polypeptide N alone, because of the drastic method of hydrolysis employed by them (16% HCl for 20 hours) which would lead to the hydrolysis of some protein. Presumably, although no experimental evidence has so far been obtained the bulk of this fraction is composed of dipeptides and lower polypeptides. Whatever may be the composition of this fraction, the experimental data point to the fact that it can on occasion play an important part as a source of nitrogen for yeast. In some cases (Table I, Series IV) as much as 30.6% of residual N is utilized by yeast.

*Protein N.* The protein N figures require little comment. The concentration of protein N is low and is scarcely attacked by yeast during the whole course of fermentation.

Summarizing these results, it can be said that the principal source of nitrogen



TABLE I

PALE ALE WORT PITCHED WITH TOP-YEAST AT THE RATE OF 2.8 G/LITRE PRESSED YEAST  
(= 1 lb/BARREL). ALL FERMENTATIONS CARRIED OUT IN DEWAR CYLINDERS

Hours	Gravity	% Total Soluble N	% Total Crystalloid N	% Ammonia N	% Amino N	% Asparagine N	% Residual N	% Protein N
<i>Series I</i>								
0	1031.5	0.064	0.047	0.0020	0.0187	0.0066	0.0197	0.017
9	1029.3	0.057	0.038	0.0018	0.0153	0.0054	0.0155	0.019
24	1014.5	0.041	0.027	0.0004	0.0091	0.0038	0.0137	0.014
48	1007.7	0.038	0.027	0.0004	0.0076	0.0048	0.0142	0.011
72	1006.9	0.039	0.027	0.0004	0.0081	0.0038	0.0147	0.012
96	1007.0	0.038	0.027	0.0004	0.0071	0.0038	0.0157	0.011
	Attenuation = 78 %							

<i>Series II</i>								
0	1040.0	0.093	0.075	0.0030	0.0308	0.0064	0.0348	0.018
9	1038.8	0.088	0.065	0.0029	0.0271	0.0058	0.0292	0.023
24	1026.2	0.064	0.051	0.0007	0.0172	0.0036	0.0295	0.013
48	1013.2	0.056	0.044	0.0005	0.0128	0.0044	0.0263	0.012
72	1011.4	0.056	0.044	0.0005	0.0128	0.0044	0.0263	0.011
96	1007.1	0.055	0.044	0.0005	0.0128	0.0044	0.0263	0.011
	Attenuation = 82.3 %							

<i>Series III</i>								
0	1039.6	0.092	0.078	0.0032	0.0308	0.0064	0.0376	0.014
9	1036.0	0.087	0.071	0.0029	0.0278	0.0044	0.0359	0.016
24	1021.2	0.068	0.057	0.0013	0.0181	0.0058	0.0318	0.011
48	1011.7	0.065	0.053	0.0006	0.0135	0.0070	0.0318	0.012
72	1010.3	0.065	0.053	0.0005	0.0133	0.0074	0.0318	0.012
96	1009.2	0.069	0.056	0.0005	0.0133	0.0074	0.0349	0.013
	Attenuation = 76.8 %							

*Series IV.* Bottom Fermentation. Decoction wort pitched with bottom yeast at the rate of 1.58 lb/barrel pressed yeast

0	1041.2	0.080	0.062	0.0029	0.0247	0.0086	0.0258	0.018
24	1036.9	0.075	0.055	0.0025	0.0217	0.0066	0.0242	0.020
48	1029.9	0.066	0.045	0.0017	0.0195	0.0050	0.0188	0.021
72	1014.3	0.059	0.042	0.0003	0.0153	0.0034	0.0230	0.017
96	1010.8	0.057	0.041	0.0006	0.0135	0.0056	0.0219	0.016
120	1010.7	0.057	0.041	0.0005	0.0141	0.0058	0.0206	0.016
144	1010.4	0.057	0.041	0.0008	0.0147	0.0046	0.0209	0.016
168	1010.4	0.057	0.038	0.0008	0.0144	0.0032	0.0196	0.019
240	1010.3	0.057	0.038	0.0009	0.0148	0.0044	0.0179	0.019
	Attenuation = 75 %							

supply for yeast growth in wort is amino acid N with residual N playing a subsidiary role. Ammonia N is present in relatively low concentration (3-4% of the total N content), but it is practically entirely absorbed by top-fermentation yeasts (84-86%), whereas bottom yeasts, after a heavy initial absorption, apparently excrete ammonia N. Asparagine N, although the best single source of nitrogen for yeasts (cf. THORNE<sup>15</sup>), apparently plays a minor role as a source of nitrogen for yeast growth in wort.

#### IV. THE FATE OF INDIVIDUAL AMINO ACIDS IN TOP FERMENTATION

Up to the present time, only six amino acids have been recognized with any certainty in wort, namely, leucine, tryptophan, tyrosine, aspartic acid (as its amide asparagine), arginine and histidine (cf. BROWN<sup>2</sup> and MISKOVSKY<sup>3</sup>). The nature and fate of free individual amino acids present in wort has been re-examined in this investigation. Eighteen individual amino acids have been recognized by partition chromatography and microbiological assay, and the fate of sixteen quantitatively followed in fermentation. As has already been mentioned the greater number of the results are concerned with top-fermentation yeasts and the figures for the behaviour of sixteen individual amino acids in fermentation are shown in Table II. Figures were also obtained for a bottom-fermentation, but in view of the fact that a number of differences became apparent between the two types of fermentation, these are separately discussed below.

From the results given in Table II, the behaviour of five amino acids requires special attention, namely, methionine, lysine, aspartic acid, leucine and proline. In the case of lysine and methionine, 59% of the former and 33% of the latter are removed from wort in 9 hours of fermentation, while in 24 hours the whole of the methionine disappears and 87% of the lysine, aspartic acid and leucine. The behaviour of lysine is of particular interest, because when supplied alone as a nitrogen nutrient for yeast it is quite useless for growth (cf. THORNE<sup>15</sup>). In other words, yeast is apparently unable to deaminate lysine by the ERLICH mechanism when it is supplied as a single source nitrogen nutrient, but when it is present in a mixture of amino acids it is selected before all others for attack. The behaviour of yeast towards proline is also of interest. Proline in wort is scarcely attacked by yeast, but it has been shown by THORNE<sup>15</sup> that proline as a single nitrogen nutrient for yeast gives rise to good growth. In fact the reverse of the behaviour of lysine occurs.

With regard to the behaviour of amino acids as a whole during fermentation a definite sequence of reactions can be seen. Yeast utilizes the so-called straight-chain or aliphatic amino acids first, *e.g.*, methionine, lysine, leucine, aspartic acid, isoleucine, *etc.*, and then proceeds to attack the ring or aromatic amino acids, *e.g.*, phenylalanine, tyrosine, tryptophan and histidine, while proline (which is also a ring compound) is scarcely affected. The behaviour of two further amino acids, tryptophan and histidine also requires consideration. Tryptophan alone is a poor source of nitrogen for yeast, while histidine is practically useless (cf. THORNE<sup>15</sup>) yet when present in a mixture of amino acids, both are taken up to the extent of 90%.

The incidence and fate of glycine (aminoacetic acid) and  $\alpha$ -alanine ( $\alpha$ -aminopropionic acid) in wort could not be followed quantitatively, because no methods of microbiological assay have so far been devised for their determination. In this instance resort was had to "partition chromatography" (cf. CONSDEN, GORDON, AND MARTIN<sup>11</sup>). The

TABLE II  
PALE ALE WORT PITCHED WITH TOP-YEAST AT THE RATE OF 2.8 g/LITRE PRESSED YEAST (= 1 lb/BARREL)  
ALL FERMENTATIONS CARRIED OUT IN DEWAR CYLINDERS

Hours Gravity	1st Experimental Fermentation					2nd Experimental Fermentation					3rd Experimental Fermentation							
	0 1039.6	9 36.7	24 16.3	48 10.8	72 10.0	96 10.1	0 1040	9 36.7	24 21.4	48 10.7	72 9.0	96 8.3	0 1040	9 38.8	24 26.2	48 13.2	72 11.4	96 7.1
Amino acids in mg/100 ml																		
Arginine	25.00	23.20	7.20	1.75	1.75	1.75	25.00	17.00	4.80	1.75	1.75	1.75	26.80	25.40	6.30	0.84	0.78	0.78
Aspartic acid	6.15	4.70	0.65	0.52	0.52	0.52	5.50	4.50	0.65	0.52	0.52	0.52	10.00	10.00	1.62	0.63	0.60	0.60
Cystine	1.50	1.50	1.25	0.50	0.50	0.50	1.60	1.50	0.55	0.55	0.55	0.55	0.95	0.95	0.90	0.45	0.45	0.45
Glutamic acid	14.10	10.00	5.00	2.80	2.80	2.80	13.20	11.00	5.00	3.20	2.60	2.60	16.50	15.50	4.60	2.00	2.00	2.00
Histidine	4.90	4.20	0.90	0.77	0.75	0.75	5.30	4.50	2.15	1.15	1.00	1.00	4.00	3.90	1.68	0.76	0.60	0.47
Isoleucine	8.60	7.00	0.87	0.82	0.82	0.82	8.50	8.20	1.55	1.31	1.20	1.13	12.00	10.50	2.20	0.67	0.60	0.60
Leucine	13.80	12.50	1.27	1.41	1.13	1.13	15.00	13.00	1.90	1.75	1.18	1.18	18.20	16.40	3.20	0.93	0.84	0.84
Lysine	15.00	5.40	1.57	2.70	2.00	2.00	18.00	5.65	3.00	3.00	3.60	3.60	7.55	4.20	0.80	0.72	0.72	0.72
Methionine	2.80	1.52	0.00	0.00	0.00	0.00	3.10	2.07	0.00	0.00	0.00	0.00	3.90	3.10	0.00	0.00	0.00	0.00
Phenylalanine	5.30	5.30	0.50	0.32	0.32	0.31	7.00	6.20	2.15	0.40	0.40	0.40	11.10	11.10	4.00	0.69	0.67	0.67
Proline	50.00	50.00	41.50	40.00	40.00	40.00	47.50	47.50	47.00	41.60	41.60	41.60	49.00	49.00	48.00	44.00	44.00	44.00
Serine	7.50	5.00	1.85	0.90	1.40	1.40	8.00	6.70	2.85	1.43	1.43	1.43	7.40	6.50	1.52	1.15	1.26	1.26
Threonine	10.80	7.40	1.38	1.25	1.20	1.20	10.80	8.20	2.25	1.22	1.20	1.20	9.40	9.00	2.45	0.80	0.66	0.66
Tyrosine	5.00	5.00	1.50	0.30	0.60	0.60	10.60	9.00	7.50	1.40	1.25	1.00	10.40	10.40	6.00	0.64	0.30	0.21
Tryptophan	6.00	5.60	0.76	0.00	0.00	0.00	2.80	2.76	0.58	0.17	0.07	0.07	5.20	4.80	4.65	0.20	0.11	0.13
Valine	14.60	12.40	1.44	1.02	1.02	1.02	13.50	12.30	3.50	1.43	1.43	1.43	18.90	18.90	6.00	0.88	0.75	0.75

results showed that glycine and  $\alpha$ -alanine are present in wort and both are absorbed by yeast. It must be emphasized that these results are purely qualitative; nevertheless, judging by eye from the separation chromatograms of other amino acids before and after fermentation, glycine and  $\alpha$ -alanine are absorbed to the extent of 80–90%. The fact that glycine when present in wort is a nitrogen nutrient for yeast, furnishes yet another example of the difference in metabolic behaviour of this organism when supplied with a mixture of nitrogen nutrients compared with a single source of nitrogen, because THORNE<sup>15</sup> has shown that when used alone, glycine is useless as a nitrogen nutrient for yeast.

#### V. METHIONINE ASSIMILATION BY YEASTS

The fact that *all* methionine is removed in the course of 24 hours fermentation by top yeasts strongly suggests that this amino acid must play an important role in the nitrogen metabolism of this type of yeast. Methionine is a sulphur-containing amino acid  $\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CH(NH}_2\text{)COOH}$  (methyl-thiol- $\alpha$ -amino-*n*-butyric acid) and is one of the ten "essential" amino acids of W. C. ROSE<sup>16</sup> for mammalian nitrogen metabolism. Since methionine appears to play a leading part in the nitrogen metabolism of top yeasts, experiments were carried out to see whether a top yeast will take up a further supply of this acid after 24 hours fermentation. This was found to be the case (Table III).

TABLE III

	Hours fermentation	Methionine Content mg/100 ml
Wort	0	2.8
"	24*	0.0 (+ 4.0 mg/100 ml methionine)
"	48	0.0

\* Methionine added at the rate of 4.0 mg/100 ml

Moreover, if the methionine content of a wort be doubled by fortification, the whole of the methionine again disappears in 24 hours (Table IV).

TABLE IV

	Hours fermentation	Methionine Content mg/100 ml
Wort	0	3.7
Wort + methionine	0	7.6
Wort + methionine	24	0.0

Thus a top yeast appears to have a very high capacity for absorbing methionine from wort in the early stages of fermentation.

In view of the results given above, other types of yeast, *e.g.*, bottom fermentation yeasts, bakers' yeast, a wine yeast and wild yeast were examined for their behaviour towards methionine. The results are given in Table V.

TABLE V

DIFFERENT TYPES OF YEAST GROWN ON A PALE ALE WORT. ALL YEASTS PITCHED AT 18.3° C AND AT THE RATE OF 3.7 g/LITRE SPUN YEAST. METHIONINE ESTIMATED AFTER 24 HOURS

	Methionine Content mg/100 ml
Wort (Original Gravity 1040)	3.50
Wort after 24 hours fermentation with:	
Baker's Yeast (1) . . . . .	0.00
Baker's Yeast (2) . . . . .	0.00
Wine Yeast (ex HELM) . . . .	0.00
Bottom Yeast (TUBORG) (1) . .	0.54
Bottom Yeast (TUBORG) (2) . .	0.54
<i>S. Macedoniensis</i> . . . . .	1.00
<i>S. festinans</i> . . . . .	0.57
<i>S. exiguus</i> . . . . .	1.00
<i>Torula</i> (sp.) . . . . .	1.55
Wild yeast (unknown origin)	1.04

The figures given in Table V show that it is only bakers' yeast (which is a top yeast) and a wine yeast (which in this particular case is also a top yeast) behave in the same way as top-fermentation brewery yeasts, *i.e.*, absorb all methionine in 24 hours. On the other hand, bottom yeasts and wild yeasts only gradually absorb this amino acid, although they were given the relatively high temperatures of a top-fermentation system. This question of the difference in behaviour towards methionine of these two main types of brewery yeasts (*i.e.*, top and bottom yeasts) was further investigated by following the fate of methionine in a true bottom-fermentation (Table VI).

TABLE VI  
DECOCTION WORT (ORIGINAL GRAVITY 1045)

Sample	% Total N	% amino acid N	Methionine Content mg/100 ml
0 days	0.070	0.024	2.80
1 day	0.063	0.021	1.52
2 days	0.053	0.016	0.44
3 "	0.048	0.014	0.38
4 "	0.046	0.015	0.30
5 "	0.048	0.014	0.25
6 "	0.048	0.014	0.20
7 "	0.044	0.013	0.15
8 "	0.046	0.013	0.12
9 "	0.044	0.014	0.12
10 "	0.044	0.014	0.12

We are indebted to Dr E. HELM of the *Jorgensen Laboratory, Copenhagen*, for these samples.

This experiment fully bears out the results given in Table V and shows that whether a fermentation be carried out with a bottom yeast at the low temperatures of lager brewing, or the comparatively high temperatures of top yeast fermentation, the result is the same and methionine is only gradually absorbed.

*References p. 691.*

## VI. THE FATE OF INDIVIDUAL AMINO ACIDS IN BOTTOM FERMENTATION

The fate and behaviour of the same 16 amino acids which were determined for top fermentations (see Table II) are shown for a bottom fermentation in Table VII.

TABLE VII

DECOCTION WORT PITCHED WITH BOTTOM YEAST AT RATE OF 1.58 lb/BARREL PRESSED YEAST.  
FERMENTATION CARRIED OUT UNDER BOTTOM-FERMENTATION CONDITIONS

Amino acids in mg/100 ml	Hours								
	0	24	48	72	96	120	144	168	240
Arginine	18.00	12.50	9.30	5.64	4.95	4.80	4.65	4.27	4.65
Aspartic acid	6.56	5.12	3.48	1.02	0.66	0.66	0.66	0.66	0.66
Cystine	0.83	0.83	0.75	0.49	0.49	0.49	0.30	0.30	0.30
Glutamic acid	14.40	10.84	10.00	3.79	2.35	2.25	5.00	7.00	7.00
Histidine	3.30	3.00	2.10	1.86	1.26	1.26	1.26	1.26	1.26
Isoleucine	11.10	9.10	7.20	3.60	1.30	1.30	1.30	1.30	1.30
Leucine	15.65	12.50	7.80	3.72	1.40	1.40	1.62	1.60	1.62
Lysine	6.35	3.00	1.10	0.78	0.30	0.30	0.30	0.30	0.30
Methionine	3.0	1.90	0.76	0.30	0.30	0.30	0.30	0.30	0.30
Phenylalanine	11.50	11.00	8.00	1.20	0.95	0.93	0.95	1.00	1.00
Proline	63.00	63.00	63.00	59.00	59.00	59.00	59.00	59.00	59.00
Serine	8.00	6.20	4.55	1.74	1.48	1.48	1.48	1.48	1.48
Threonine	8.25	5.10	3.00	2.20	1.50	1.50	1.50	1.50	1.50
Tyrosine	12.60	11.00	9.00	4.60	2.32	1.80	1.65	1.65	1.65
Tryptophan	5.84	5.20	4.80	2.50	1.98	1.98	1.98	1.98	1.98
Valine	15.50	13.60	10.30	3.28	2.32	2.32	2.32	2.64	2.40

Certain similarities and differences are apparent when the results in the two cases are compared. In the first place the behaviour of lysine and proline is exactly the same for both types of fermentation. 50% of the lysine is removed in the first 24 hours of fermentation and in all, 95% is taken up by the bottom yeast. Proline, as in top fermentation, is scarcely attacked and methionine, although utilized to the extent of 90% is only gradually absorbed. The behaviour of glutamic acid is markedly different from a top-fermentation. The bottom yeast removes 84% of this acid in 120 hours and thereafter there is vigorous excretion up to approximately 50% of the original glutamic acid content of the wort. No other amino acid is excreted to this extent in either a top or bottom-fermentation. In top-fermentations the aromatic amino acids, tryptophan and histidine are used up to 90%, but in a bottom-fermentation only about 65% of these acids are removed.

With the exception of aspartic acid, leucine, lysine, methionine, phenylalanine and tyrosine, which are taken up to the extent of 90%, the remaining acids are not absorbed by a bottom yeast to the same degree as they are in a top-fermentation.

Thus, there are several differences to be noted in these two types of fermentation. How far they are due to differences in the temperatures of fermentation and how far to inherent differences in the yeasts themselves is impossible to say at the present time.

## VII. THE AMINO ACID CONTENT OF YEAST PROTEIN

It has been known for many years that yeasts are able to use inorganic nitrogen, *e.g.*, ammonium sulphate or phosphate as their sole source of nitrogen and are able

to synthesize from such relatively simple compounds the complex proteins of their protoplasm. Generally speaking, however, they prefer elaborated organic nitrogen and show greatly enhanced growth on a medium containing a mixture of amino acids compared with a medium containing a single source of nitrogen, *e.g.*, an ammonium salt or single amino acid (cf. THORNE<sup>17</sup>). It was therefore of interest to determine if any differences were produced in the amino acid content of yeast protein by growing the organism in media containing different types of nitrogen. Yeast protein is considered "first class" protein and in this respect is comparable with the best animal proteins, *e.g.*, casein. A protein is considered "first class" when it contains high values of the following 10 "essential" amino acids of ROSE<sup>16</sup>, arginine, histidine isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Table VIII shows the values for seven "essential" acids in yeast protein.

The yeast was grown on a variety of different media and for comparative purposes the values for the same acids in casein are also given.

The figures given in Table VIII show that the values of the seven acids estimated in yeast protein compare favourably with the same acids in casein, with the exception of methionine which is low. Apart from methionine, the figures for the remaining acids are remarkably constant whatever the nature of the medium upon which the yeast has been grown. In the case of methionine, however, it is possible to increase its concentration in yeast protein as a result of increasing its concentration in the medium (Table VIII, No. 7). No other case of increase was found among the other six acids although medium No. 7 (Table VIII) was also fortified with histidine and phenylalanine.

#### VIII. DISCUSSION

The main points of the present investigation can conveniently be discussed here. The behaviour of asparagine N in fermenting wort requires consideration. It is difficult to understand why the best single source of nitrogen nutrient for yeast becomes only of minor importance when present in wort. The evidence for excretion of asparagine N during fermentation is abundantly plain. The suggestion is tentatively made here that the excretion of this amide during fermentation may be bound up in some way with the neutralization of any excess ammonia formed in the yeast cell during its metabolic activities, which might otherwise prove toxic to the organism.

Hitherto, a mixture of amino acids has been considered to be the best source of nitrogen for yeast yielding maximum growth. In general terms this is true, but the role of residual N in this connection must also be taken into account. The composition of this fraction is not known at present, but it is highly probable that di- and lower polypeptides largely enter into its composition. If this be the case, then di- and polypeptides form a subsidiary but nevertheless significant source of nitrogen for yeast. THORNE<sup>17</sup> has shown that with increasing number of nitrogen nutrients in a medium there is a progressive enhancement of growth and he has calculated that there is a limiting value to this augmentation process of about 50%. THORNE was mainly interested in media containing mixtures of amino acids. In the present investigation THORNE's results have been confirmed (Table IX), but at the same time it must be pointed out, that the amount of growth even with a mixture containing a large number of amino acids, *e.g.*, casein hydrolysate, is always inferior to wort and it is only when peptides (peptone was used for this purpose) are added that growth in an artificial medium and growth on wort

TABLE VIII  
TOP YEAST PITCHED AT RATE OF 3.7 g/LITRE SPUN YEAST

Yeast grown on	Methionine		Phenylalanine		Histidine		Lysine		Leucine		Isoleucine		Valine	
	% Dry wt	16% N	% Dry wt	16% N	% Dry wt	16% N	% Dry wt	16% N	% Dry wt	16% N	% Dry wt	16% N	% Dry wt	16% N
Wort (O.G. 1040). . . . .	0.70	1.48	1.61	3.42	1.30	2.77	3.20	6.80	3.00	6.38	2.44	5.18	2.93	6.40
Wort (O.G. 1046). . . . .	0.89	1.45	1.93	3.14	1.52	2.48	4.37	7.12	3.78	6.16	3.07	5.00	3.92	6.40
Artificial medium + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.73	1.43	2.05	4.00	1.73	3.40	3.48	6.84	3.05	6.00	2.73	5.36	3.09	6.08
Artificial medium + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + Asparagine . . . . .	0.67	1.43	1.82	3.85	1.40	3.00	3.48	7.37	3.27	6.95	2.73	5.78	3.09	6.55
Artificial medium + peptone	0.57	1.16	1.62	3.28	1.07	2.17	3.64	7.37	2.76	5.59	2.28	4.62	2.62	5.30
Artificial medium + peptone and casein hydrolysate . .	0.76	1.53	2.00	4.04	1.30	2.64	4.34	8.76	3.13	6.33	2.52	5.10	2.91	5.90
Artificial medium + casein hydrolysate fortified with methionine phenylalanine and histidine . . . . .	1.73	3.50	1.42	2.88	1.15	2.33	3.26	6.58	2.85	5.75	2.31	4.66	2.54	5.13
Casein <sup>1</sup> . . . . .	—	3.00	—	5.20	—	2.50	—	6.90	—	12.10	—	6.50	—	7.00

<sup>1</sup> Values taken from R. J. BLOCK AND D. BOLLING<sup>18</sup>.



become identical. Care was taken in the preparation of the artificial media that gravity and the total nitrogen content were as nearly as possible the same in all cases as the wort which served as control.

TABLE IX  
TOP-YEAST USED AND PITCHED AT THE RATE OF 2.8 g/LITRE PRESSED YEAST (= 1 lb/BARREL)

Nitrogen constituent of artificial medium	% Total N	Original Gravity	Present Gravity	Reproduction (g/litre = spun wet weight)
1. Ammonium sulphate . . . . .	0.074	1032.0	1001.9	11.8
2. Ammonium sulphate + asparagine .	0.079	1032.4	999.9	19.6
3. Peptone . . . . .	0.082	1032.7	1000.2	24.3
4. Casein hydrolysate . . . . .	0.073	1033.0	998.2	20.8
5. Casein hydrolysate + peptone . . .	0.078	1033.1	999.4	26.0
6. Wort . . . . .	0.072	1032.5	1005.5	25.4

This experiment has been repeated and confirmed many times. Thus it would appear that peptides as a source of nitrogen for yeast cannot be neglected in forming a general picture of the subject.

The question of the absorption of individual amino acids from wort has already been discussed, and the similarities and differences between top and bottom-fermentation yeast pointed out.

I wish to thank the Directors of Messrs WHITBREAD AND Co Ltd for permission to publish this investigation. I would also like to take this opportunity of thanking Mr B. M. BROWN for his helpful criticism and advice throughout the course of this work. I am grateful to my assistants Mr N. S. CURTIS and Miss J. V. SUTTON for their help in the practical work of this investigation.

#### SUMMARY

A quantitative examination of the more important nitrogenous groupings present in wort, *e.g.*, ammonia N, amino acid N, asparagine N, residual N and protein N have been made, and their fate in fermentation by top and bottom-yeasts followed.

The presence of 18 individual free amino acids has been detected in wort and the behaviour of 16 during fermentation by top and bottom-yeasts followed by microbiological assay.

Seven "essential" amino acids in yeast protein have been determined. The yeast was grown on media containing different sources of nitrogen. With the exception of methionine, all the acids were constant in amount whatever the nature of the medium. The concentration of methionine in yeast protein could be increased by increasing its concentration in the medium.

#### RÉSUMÉ

Un examen quantitatif des groupements azotés importants présents dans le moût (azote ammoniacal, des acides aminés, de l'asparagine, de protéine et azote résiduel) a été fait et le sort en a été suivi au cours de la fermentation haute et basse.

La présence dans le moût de 18 acides aminés libres a été décelée; le comportement de 16 de ceux-ci pendant la fermentation haute et basse a été suivi par estimation microbiologique.

Sept acides aminés "essentiels" de la protéine de la levure ont été déterminés. La levure a été cultivée sur milieux contenant différentes sources d'azote. À l'exception de la méthionine, tous les acides se trouvaient à concentration constante, quelle que fût la nature du milieu. La concentration de la méthionine dans la protéine de levure pouvait être augmenté en augmentant sa concentration dans le milieu.

References p. 691.

## ZUSAMMENFASSUNG

Die wichtigeren stickstoffhaltigen Gruppen, welche in der Würze vorkommen, wie Ammoniak-N, Aminosäure-N, Asparagin-N, Protein-N und Rest-N wurden quantitativ untersucht, und ihr Schicksal während der Ober- und Untergärung wurde verfolgt.

Die Anwesenheit von 18 freien Aminosäuren in der Würze wurde festgestellt und das Verhalten von 16 dieser Säuren während der Vergärung durch Ober- und Untergärhefen wurde mikrobiologisch verfolgt.

Sieben für das Hefeeiweiss "wesentliche" Aminosäuren wurden festgestellt. Die Hefe wurde auf Nährböden, welche verschiedene Stickstoffquellen enthielten, gezüchtet. Mit Ausnahme des Methionins war die Menge aller Säuren konstant, was auch die Art des Nährbodens sein mochte. Die Konzentration des Methionins im Hefeeiweiss konnte durch Erhöhung der Methioninkonzentration im Nährboden gesteigert werden.

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## GROWTH AND FERMENTATION FACTORS FOR DIFFERENT BREWERY YEASTS\*

by

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Brewery fermentation is a complex process which involves both yeast growth and alcoholic fermentation, in addition to such other changes as are concerned with flavour. The reactions which occur may be studied by an analytical method, or a synthetic procedure may be used in which an attempt is made to reproduce the principal features of brewery fermentation in solutions of known composition. Eventually, it may be necessary to utilize fully both types of approach before enough is learned of the process to control it.

Recently, the problem has been attacked in our laboratories from the synthetic point of view. In an effort to study, separately at first, the factors which yeast requires for growth and fermentation, conditions and media were chosen such as would yield, in the former instance, maximal growth with little fermentation and then the reverse. Finally, to investigate those factors under conditions more closely related to actual brewery practice, conditions were set up intermediate between the two in which appreciable growth as well as fermentation occurs.

This work, here to be reported, hence consists of three phases:

1. A study of the bios requirements of various pure cultures of brewery yeasts for reproduction, growth being followed in a series of solutions at 30° C.
2. A study of initial rates of fermentation by pressed brewers' yeast in synthetic media as compared with wort.
3. Finally, an investigation of the combined processes of growth and fermentation under brewery fermentation conditions.

In the first study, the growth requirements of brewers' yeast cultures were studied under conditions designed to show the ability of the various organisms to synthesize the several bios or growth factors<sup>1</sup>. These tests were conducted at 30° C in shallow layers of media at a minute seeding rate. The extent of growth was measured turbidimetrically at the end of 40 hours. These studies disclosed that brewers' yeast strains differed as regards their bios requirements so that a number of different types could be identified. Types were shown to be relatively quite stable in respect to the particular factors required for growth. Thus it became possible to identify certain specific yeast cultures even after several years' use, in the brewery. Differences among brewery yeasts thus disclosed may be of value in the introduction of new cultures and in the maintenance of culture purity when, as is common brewing practice, yeast is used for many cycles.

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In the second phase of our study dealing with fermentation rates, principal attention was paid to the fermentation kinetics as measured by the rate of  $\text{CO}_2$  evolution during the first few hours of fermentation carried out at  $30^\circ \text{C}^2$ . A medium was developed in which the initial rate of fermentation, using a relatively high yeast inoculum, is equal to that in beer wort. The various components of this synthetic solution were then investigated as to their individual influence on the rate, and their optimum concentrations were determined. Certain differences were found in the response by brewers' yeast to components of the synthetic solution as compared with the responses previously reported for bakers' yeast.

Normal brewery fermentations are conducted at low temperatures, relatively low seeding rates, and in deep vessels — circumstances which result in essentially anaerobic conditions in a short time. Although the principal reaction is alcoholic fermentation, a significant degree of yeast growth nevertheless takes place. For the successful production of beer, both processes appear essential. Therefore, in the third phase of our work, the synthetic medium was fermented at a temperature ( $12^\circ \text{C}$ ) comparable to brewery conditions and at a seeding rate corresponding to normal brewing practice. Under these conditions, using pressed brewery yeast, it was found that, unlike indications from the previous fermentations, the addition of a mixture of bios factors was needed before the synthetic solution could be made to ferment at a rate approaching that obtained with wort. On further examination, it was found that one of the bios factors, inositol, is the main factor for maximal fermentation rate under such conditions. It would seem that yeast, as taken from a normal brewery fermentation, has insufficient stores of inositol to permit it to grow in, and ferment, at an optimal rate, a synthetic solution deficient in this factor. Various aspects of the inositol requirement were studied, notably, the significance of this factor for two yeasts differing as to their need for inositol for growth. The effective inositol concentration also was determined and compared with the amount of this bios factor present in beer wort.

#### GROWTH FACTORS

Yeasts, in common with a great many other organisms, require more than pure sugar and salts for development of maximum growth rates. To obtain the growth and reproduction ordinarily observed in plant extracts such as beer wort, grape juice, etc., one or more of a group of substances known as bios factors (WILDIERS') must, it is now well known, be present. These factors are inositol, pantothenic acid, biotin, thiamin, and pyridoxine. Some yeasts, principally lactose fermenters<sup>3</sup>, also are reputed to require nicotinic acid.

It has been shown by a number of investigators<sup>4,5,6,7</sup> that yeasts differ markedly in the number and combination of bios factors required. Cultures obtained from various national collections of type cultures showed marked differences in their bios requirements even among members of groups presumably closely related in their industrial uses (*e.g.*, distillers' yeasts, etc.) and which were so similar as to be indistinguishable by ordinary taxonomic techniques. The success of industrial fermentations depends greatly, it is generally believed, on the nature and the constitution of the particular strains of the microorganism in use. Hence, the potential practical significance of an increase in specificity of classification methods for the fermentation industries should be obvious.

Because variation, mutation, and segregation have been reported as occurring in yeast cultures under one condition or another<sup>8, 9</sup> it was considered necessary to observe the constancy of the growth characteristics of the various cultures involved. In addition to the usual storage on agar slants, the cultures were subjected to various other treatments which were likely to be encountered in commercial practice and which might conceivably cause mutation to occur. The yeasts were then re-isolated (multiple isolates) and single cell isolates tested for their bios requirements under standardized conditions as described.

#### *Methods and Apparatus*

The medium is based on that used in microbiological vitamin assay techniques using yeast as the test organism<sup>10</sup>. The composition of this medium as finally adopted is shown in Table I. In practice, a series of more concentrated solutions are prepared containing a) the basal medium without bios factors, b) the five bios factors, and c), d), e) and f) bios solutions deficient in one or more of the factors. These solutions are preserved in the refrigerator under a preservative<sup>11</sup> composed of n-butylchloride (three volumes) and carbon tetrachloride (one volume).

TABLE I  
COMPOSITION OF SYNTHETIC GROWTH MEDIUM

Dextrose, c.p. (anhydrous) . . . . .	50	g	per 1000 ml
Potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) . . . . .	0.55	g	„ „ „
Potassium chloride (KCl) . . . . .	0.425	g	„ „ „
Calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) . . . . .	0.125	g	„ „ „
Magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) . . . . .	0.125	g	„ „ „
Ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) . . . . .	2.5	mg	„ „ „
Manganese sulphate ( $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ) . . . . .	2.5	mg	„ „ „
Potassium citrate buffer, pH 5.5 (0.4 M) . . . . .	50	ml	„ „ „
Casein hydrolysate (vitamin-free) 8%, pH 5.5 . . . . .	50	ml	„ „ „
Inositol . . . . .	25	mg	„ „ „
Calcium pantothenate . . . . .	2.5	mg	„ „ „
Biotin . . . . .	0.025	mg	„ „ „
Thiamin hydrochloride . . . . .	0.5	mg	„ „ „
Pyridoxine hydrochloride . . . . .	0.5	mg	„ „ „

The inoculum is prepared from a fresh 24-hour growth (at 30° C) of the yeast on a malt-agar slant. A wire loop of yeast is transferred to 10 ml of sterile saline contained in a colorimeter tube. The concentration of yeast is determined and adjusted with additional saline or yeast to 1 mg per ml. An aliquot of the suspension is further diluted with sterile saline to a yeast concentration of 0.1 mg per ml. One ml of this final suspension is used to inoculate each flask.

The procedure employed was as follows: The various media were made to a volume of 9 ml in each of a series of flasks, plugged with cotton, sterilized in flowing steam for 20 minutes, cooled, and inoculated with 1 ml of a suspension containing 0.1 mg of the yeast under study.

The growth tests are conducted in a volume of 10 ml of medium contained in 125 ml Erlenmeyer flasks. The flasks are incubated at 30° C. When the measurement of yeast growth is used for vitamin assay or when the medium is contained in test tubes or smaller flasks, it is generally desirable to shake the vessels in order to incorporate an adequate supply of air. With the larger flasks employed in the present work, no difference due to shaking could be observed in parallel tests. Hence the flasks were not shaken during incubation in the present studies. Yeast growth is customarily estimated at 24 and 40 hours (duplicate flasks) but in the present report, only the 40-hour results have been considered.

The estimation of yeast growth is based upon the absorption of light in the EVELYN photoelectric colorimeter (660 filter). A calibration curve relating milligrams of moist yeast to absorption per cent was initially constructed using compressed bakers' yeast as a reference standard. The yeast solids equivalent corresponding to the calibration curve was determined for a number of different brewers' yeast cultures by first estimating the moist yeast content of a suspension from the curve and then determining the yeast solids content of an aliquot of the suspension by the A.S.B.C. method. The average of four such determinations was 28.98% (standard deviation = 0.72), *i.e.*, our estimation of the concentration of moist yeast is based on a yeast of approximately 29% solids, which is about average for compressed yeasts.

References p. 708.

## EXPERIMENTAL

Yeast samples were obtained from a number of breweries and plated out on wort agar. Single colonies were selected at random and transferred to agar slants. They were replated to check purity and then stored at 5° C. A total of 58 cultures was collected in the first stage of this study. Of these, 53 represent lager (bottom) yeast cultures and the remaining 5, ale or top yeasts. All cultures were subjected to routine fermentation tests. In such tests the lager yeasts uniformly fermented melibiose whereas the ale yeasts did not.

When grown in the several media deficient in one or another of the bios factors, the various cultures showed significant differences in their ability to grow at a rate comparable to that shown in the so-called "complete" medium.

In the all factor medium, most of the cultures produced about 16 mg of moist yeast per ml in 40 hours. As a first approximation we considered that a yeast required a bios factor when growth in the absence of the factor was less than 50% of that shown by the same yeast in the all factor medium. On this basis, it is possible to group the cultures into five categories:

Those which require

1. biotin only
2. biotin and pantothenate
3. biotin and inositol
4. biotin, pantothenate and inositol
5. biotin, pantothenate, inositol and either thiamin or pyridoxine.

The growth of five different yeasts, representative of each of these types, is shown in Fig. 1, and the growth responses of the yeasts studied are summarized in Table II; here, the range of growth in the various media is given for all of the cultures in each group. It might be well to emphasize that the upper and lower figures for the range of growth in the table represent different yeasts and not results of replicate tests with the same culture. It will be observed that all of the yeasts require biotin, and some require either pantothenate or inositol or both. As regards thiamin and pyridoxine, the lager and ale yeasts show a sharp division. The lager yeasts were found to grow well when both thiamin and pyridoxine were simultaneously omitted from the medium. On the

TABLE II  
BIOS REQUIREMENTS OF BREWERS' YEAST GROUPS  
RANGE OF GROWTH — MG MOIST YEAST PER ML AT 40 HOURS (30° C)

Cultures	Required Factors	Growth Range	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B <sub>1</sub> & B <sub>6</sub> Omitted
14 Lager	Biot.	min	12.2	0.0	16.9	7.7	10.4
		max	15.0	0.5	13.2	14.4	17.2
23 Lager	Biot., Panto.	min	11.4	0.1	0.2	7.6	12.6
		max	15.0	0.6	7.4	15.0	16.0
4 Lager	Biot., Inos.	min	12.8	0.1	8.5	1.3	12.0
		max	16.0	0.3	16.0	6.4	16.0
12 Lager	Biot., Panto., Inos.	min	12.2	0.2	0.0	0.6	10.8
		max	15.0	0.8	4.8	6.1	15.0
5 Ale	{ Biot., Panto., Inos., B <sub>1</sub> or B <sub>6</sub> }	min	12.1	0.1	0.0	0.0	1.0
		max	13.6	0.2	0.1	1.0	5.5

other hand, the ale yeasts required either thiamin or pyridoxine and grew very poorly when both were omitted. This curious apparent equivalence of pyridoxine and thiamin has been previously reported by SCHULTZ *et al.*<sup>4</sup> in studies with a variety of bakers' yeasts.

Since in the case of lager yeasts, each requires biotin and none requires either thiamin or pyridoxine, there are four possible types, *i.e.*, lager strains which in addition to biotin:

1. require no other factor
2. require pantothenate
3. require inositol
4. require pantothenate and inositol.

As may be seen, representatives of each of these groups have been found among lager yeasts.

The reproducibility of the typing method and the stability of the bios types were studied in several ways. In order to determine the influence of extended storage, for example, culture maintenance on agar slants at 5° C, the cultures so maintained were periodically examined or tested for their bios responses. The results obtained with a group of representative yeasts after storage on agar slants for periods ranging from 97 to 302 days are shown in Table III, from which it may be seen that the initial characteristics were maintained without marked alteration. In the case of culture 6C56, it

TABLE III

INFLUENCE OF EXTENDED STORAGE AT 5° C (AGAR SLANTS) ON BIOS REQUIREMENTS OF BREWERS' YEASTS  
GROWTH AT 40 HOURS (30° C) — MG MOIST YEAST PER ML

Culture	Required Factors	Storage (days)	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B <sub>1</sub> & B <sub>6</sub> Omitted
Lager 7C8	Biot.	0	13.6	0.3	11.4	12.2	13.6
		188	14.4	0.2	9.2	12.8	14.4
Lager 6C13	Biot., Panto.	0	16.8	0.2	0.2	13.7	16.1
		302	14.4	0.2	0.8	13.2	14.4
Lager 7C30	Biot., Inos.	0	16.0	0.2	16.0	1.3	16.0
		178	16.0	0.2	16.0	0.8	16.0
Lager 6C56	Biot., Panto., Inos.	0	11.7	0.2	0.1	4.4	11.7
		226	13.2	0.2	0.2	0.8	12.8
Ale 6C3	{ Biot., Panto., Inos., B <sub>1</sub> or B <sub>6</sub> }	0	12.1	0.1	0.1	0.3	1.0
		97	14.0	0.0	0.0	0.1	2.4
Bakers' 6C1	Biot., Panto., B <sub>1</sub> or B <sub>6</sub>	0	13.4	0.3	0.1	8.4	0.1
		97	14.0	0.6	0.0	8.1	0.6

may be observed that the ability to grow slightly without inositol appears to have been lost or weakened after extended storage.

As a further test of the constancy, and incidentally the reproducibility, of bios type, a number of yeasts were subjected to daily serial transfer, week ends excepted, *i.e.*, the culture was transferred daily from agar slant to agar slant, being incubated between transfers at 30° C. Under these conditions, the yeast is forced to undergo rapid, extensive multiplication at relatively high temperatures for a considerable period of time.

The results of this study are shown in Table IV; the number of serial transfers ranged from 13 to 32. It may be seen that again the basic bios characteristics have been

TABLE IV  
INFLUENCE OF DAILY SERIAL TRANSFER ON BIOS REQUIREMENTS  
GROWTH AT 40 HOURS (30° C) — MG MOIST YEAST PER ML

Culture	Required Factors	Number of Transfers	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B <sub>1</sub> & B <sub>6</sub> Omitted
Lager 7C8	Biot.	0	13.6	0.3	11.4	12.2	13.6
		28	14.4	0.0	14.0	13.2	14.4
Lager 6C13	Biot., Panto.	0	16.8	0.2	0.2	13.7	16.1
		32	11.0	0.2	0.2	8.6	11.4
Lager 6C56	Biot., Panto., Inos.	0	10.6	0.2	0.2	2.3	10.8
		13	12.2	0.2	0.0	0.3	12.2
Ale 6C3	{ Biot., Panto., } { Inos., B <sub>1</sub> or B <sub>6</sub> }	0	12.1	0.1	0.1	0.3	1.0
		32	14.4	0.0	0.0	6.2	3.4
Bakers' 6C1	{ Biot., Panto., } { Inos., B <sub>1</sub> or B <sub>6</sub> }	0	13.4	0.3	0.1	8.4	0.1
		13	13.6	0.6	0.0	6.2	0.4

maintained, although in several instances there appears to have been a weakening of the culture. This weakening appears in a reduction of the extent of growth on the all factor medium and also a reduction of the extent of growth on one or more of the deficient media.

A test to disclose possible mutation was the typing of yeasts before and after fermentation of hopped brewery wort at 10° C (simulating brewery conditions). This test was conducted as follows: The culture was brought up in increasing volumes of brewery wort and finally seeded into two liters of hopped brewery wort at 10° C where it was allowed to end-ferment (10 days) and then permitted to rest under the beer for another five days, following which it was plated out and six colonies were selected at random from each of the plates for subculture. These cultures were then tested for their bios type, with results which may be seen in Table V, where the results obtained initially on the culture and the results obtained on the first two of the isolates are compared. It will be noted that the cultures were all recovered with very little change.

TABLE V  
RECOVERY OF BIOS TYPES AFTER FERMENTATION OF BREWERY WORT AT 10° C  
GROWTH AT 40 HOURS (30° C) — MG MOIST YEAST PER ML

Culture	Required Factors	Growth	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B <sub>1</sub> & B <sub>6</sub> Omitted
Lager 7C35	Biot.	Init.	14.4	0.2	12.2	13.2	14.4
		Final*	14.4	0.2	13.2	12.8	15.0
		Final*	14.4	0.2	12.6	12.8	15.0
Lager 7C29	Biot., Panto.	Init.	14.0	0.2	0.2	13.6	14.0
		Final*	14.4	0.2	0.2	13.6	14.4
		Final*	13.2	0.3	0.2	13.2	14.0
Lager 7C30	Biot., Inos.	Init.	16.0	0.2	16.0	1.3	16.0
		Final*	17.2	0.2	17.2	2.0	18.8
		Final	17.6	0.2	16.4	1.8	18.8
Lager 7C6	Biot., Panto., Inos.	Init.	12.6	0.4	0.4	1.3	12.6
		Final*	13.2	0.3	0.4	2.0	14.4
		Final*	13.2	0.3	0.3	3.4	13.2

\* First two isolates of a series of six or more.

References p. 708.



Ever since the time of PASTEUR AND HANSEN, the importance of pure yeast culture in the art of brewing beer has been everywhere appreciated by brewers. PASTEUR first demonstrated the importance of having the yeast free from harmful bacteria and then HANSEN showed the value of specific pure yeast strains. Many a successful brewer has justifiably felt that the desired characteristics of his beer were largely due to the special properties of his particular strain. It is, therefore, perhaps not surprising that we find marked differences in the bios types of the yeasts employed in the various breweries, denoting not only different origins but also the possible effect of continued selection. Although the tests which we have thus far made do not indicate significant mutation, it is quite possible that over longer periods of time the process of mutation may have been responsible for the origin of differences in strains of brewers' yeasts.

The long range objective of research in this field is, of course, to learn how and why these various yeasts differ, one from the other, in industrial processes. In other words, it is hoped better to define the differences between them and, if possible, to determine the biochemical basis for the differences. A logical part of such a program is the study of fermentation rates in synthetic solutions, which forms the next phase of the work here presented.

#### FERMENTATION FACTORS

The principal reaction brought about by yeast in brewery wort is alcoholic fermentation, although a significant growth of yeast is necessary in each fermentation cycle if only to obtain sufficient pitching yeast for the succeeding cycle. Although, classically, fermentation has been defined as representing cell metabolism in the absence of oxygen, it is very difficult, perhaps impossible, experimentally to separate fermentation from growth when it is desired to study each activity separately. By limiting the supply of oxygen and providing elevated concentrations of yeast and sugar, one may fairly successfully observe reactions essentially fermentative in character. Reactions observed under such conditions can be described as assimilative fermentation<sup>5</sup>.

As carried out by us, one gram of moist yeast is suspended in 100 ml of medium, the mixture is shaken at 30° C, and the rate of fermentation is observed by measuring the volume of gas evolved at suitable intervals. In the course of three hours, one part of yeast converts six to eight parts of sugar. This fermentation is accompanied by a limited increase in yeast dry weight (about 20%). Thus, a predominantly fermentative reaction is established without extreme departure from the normal environment of beer yeast such as is produced by the addition of metabolic poisons like sodium azide.

Utilizing these conditions, an experimental medium was developed which incorporated factors known to favour fermentation by living yeast. The rate of fermentation in this synthetic medium was compared with the rate in beer wort. With suitable changes in composition, it was found that the rate of fermentation in such a medium (corresponding to a drop in apparent extract of about 2° Plato) was equal or superior to that observed in beer wort. Each of the components of the medium was separately investigated as to its necessity, and the concentration of each corresponding to optimal fermentation rate was determined. The composition of the final medium is given in Table VI.

The most noticeable effects were produced by the omission of  $\text{NH}_4^+$ ,  $\text{PO}_4^-$ ,  $\text{Mg}^{++}$ ,  $\text{SO}_4^-$ , and  $\text{K}^+$ . The absence of  $\text{NH}_4^+$  and  $\text{PO}_4^-$  caused a marked reduction in fermentation

TABLE VI  
SYNTHETIC FERMENTATION MEDIUM

Dextrose . . . . .	50	g	per 1000 ml
Ammonium chloride ( $\text{NH}_4\text{Cl}$ ) . . . . .	1.87	g	" " "
Sodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) . . . . .	2.48	g	" " "
Potassium chloride ( $\text{KCl}$ ) . . . . .	1.34	g	" " "
Magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) . . . . .	0.800	g	" " "
Calcium chloride ( $\text{CaCl}_2$ ) . . . . .	0.333	g	" " "
Sodium succinate buffer, pH 4.9 (M/3) . . . . .	150	ml	" " "
Casein hydrolysate (vitamin-free) 8%, pH 5.5 . . . . .	25	ml	" " "
Thiamin hydrochloride . . . . .	0.800	mg	" " "
Pyridoxine hydrochloride . . . . .	0.800	mg	" " "
Nicotinic acid . . . . .	8.00	mg	" " "

rate.  $\text{Mg}^{++}$ ,  $\text{SO}_4^{--}$ , and  $\text{K}^+$  were also necessary for the maximal rate of fermentation, although to a lesser extent. In order to accomplish the single omission of various ions, appropriate corresponding  $\text{Na}^+$  or  $\text{Cl}^-$  salts were substituted, *i.e.*,  $\text{MgCl}_2$  replaced  $\text{MgSO}_4$ , in order to observe the effect of omission of  $\text{SO}_4^{--}$ . Similarly,  $\text{Na}_2\text{SO}_4$  replaced  $\text{MgSO}_4$  in order to study the effect of absence of  $\text{Mg}^{++}$ .

In the investigation of the influence of the various ions, it was observed that a rather small excess of  $\text{Mg}^{++}$  over that necessary caused a marked depression in fermentation rate. The addition of a small quantity of  $\text{CaCl}_2$  counteracted this inhibition and augmented the stimulating effect of low concentrations of  $\text{Mg}^{++}$ . For this reason,  $\text{CaCl}_2$  was incorporated in the basic medium.

The experimental omission of thiamin, pyridoxine, and nicotinic acid had relatively little effect; nevertheless, these factors were included in the medium because, in the case of bakers' yeast, the importance of these factors had been established by other workers<sup>12</sup>. Other bios factors, biotin, calcium pantothenate, and inositol, were experimentally added without noticeable effect, most probably due to the relatively small extent of growth involved in these tests. Hence, these factors were not incorporated in the basic medium. As will be described later, when conditions were so adjusted as to favour a significant proportion of yeast growth concomitantly with fermentation, the last named factor, inositol, was found to play a very important role.

In Table VII are shown the results of an experiment in which the fermentation rate of beer wort under the above conditions is compared with fermentation in the synthetic medium as finally evolved (Table VI). Since the fermentable sugars of beer wort consist of both maltose and dextrose, experiments were made first with these separately as the sole sugars in the synthetic medium and then with a mixture of four parts of maltose and one part of dextrose. The latter mixture corresponds to the proportions we have found in samples of brewery wort by a method based upon selective fermentation by different yeast species<sup>13</sup>. It will be observed from Table VII that the rate of fermentation in the synthetic medium containing 4% of maltose and 1% of dextrose is quite equal to that observed in beer wort. In the synthetic medium in which maltose is the only fermentable sugar, the fermentation rate is considerably lower than that of wort, whereas when dextrose is the sole sugar, the rate of fermentation soon exceeds the wort rate.

This synthetic fermentation medium is obviously not in any sense a synthetic beer wort but it would appear that it contains the substances, or their physiological equivalents, necessary for a rapid initiation of fermentation at 30° C. A medium of this type might be employed as a reliable and reproducible reference standard for ascer-

TABLE VII  
RATES OF FERMENTATION IN SYNTHETIC MEDIUM AND BEER WORT

	Sugar	Gas evolved			
	Per cent w/v	60 min	120 min	180 min	240 min
Beer wort	—	68	159	285	433
Synthetic	Maltose 5	33	83	176	313
Synthetic	Dextrose 5	57	146	294	484
Synthetic	Maltose 4	67	160	287	443
	Dextrose 1				

taining possible deficiencies in beer worts or other fermentation media. A method based on this principle would be similar to the microbiological assay methods widely employed for vitamins, amino acids, etc. Conversely, such a method may be used to assess quickly the vitality and characteristics of pitching yeasts in brewery operations.

#### GROWTH AND FERMENTATION IN EXPERIMENTAL MEDIA

Having now separately studied first yeast growth and then fermentation in synthetic solutions at 30° C, it was thought desirable to extend the investigation to conditions more closely approaching those obtaining in brewery fermentation. Principal differences here are to be found in pitching rate and temperature. In order to effectively emphasize growth and fermentation in the respective studies, the growth tests had been made with a very low pitching rate (moist yeast concentration 0.01 mg per ml or 0.0026 lb per bbl). On the other hand, in the case of the fermentation tests, a pitching rate was employed equivalent to about 2.5 pounds per barrel or 10 mg per ml. An average normal pitching rate is about 0.5 lb per bbl in terms of moist or pressed yeast. This is the pitching rate which was used in the final attenuation experiments. This rate is equivalent to 1.94 mg per ml. An experimental temperature of 12° C was considered to be close to brewery fermenting temperatures. These conditions may be seen to involve a degree of growth not occurring with a pitching rate five times as great, while fermentation, at the same time, is considerably slowed both by the lower temperatures and reduced pitching rate. With these conditions as to pitching rate and temperature and using the synthetic fermentation medium shown in Table VI, the rate of attenuation was compared to that of wort. Here, the synthetic medium is found to be markedly inferior, a difference which was heightened when pitching rates were lowered further to half-normal values, *i.e.*, 1 mg per ml. Evidently, under such conditions the medium is deficient in some factor or factors present in beer wort. Experiments showed that addition to the synthetic medium of either yeast extract or a mixture of the bios factors increased the attenuation rate to that observed in wort. Inositol was disclosed as the most important of the bios factors responsible. This effect of inositol was then studied with two yeasts of differing bios types and the minimum effective concentration of inositol determined for comparison with the concentrations normally occurring in beer wort.

## METHODS AND APPARATUS

*Fermentations* were conducted in a volume of 500 ml of media contained in one-liter pyrex bottles equipped with water seals. The bottles were incubated in a constant-temperature chamber at 12° C.

*The Synthetic Medium* employed was based on that shown in Table VI with the modifications noted, i.e., the dextrose content was increased to 70 g per liter, the succinate buffer was reduced to 100 ml per liter, and additional bios factors were added as required.

*The Wort* was lager cooler wort obtained through the courtesy of a local brewery, brought to the laboratory in glass containers, filtered and boiled for three minutes in cotton-plugged flasks.

*The Yeast* was washed, compressed brewers' yeast, obtained from two local breweries, and designated for convenience as yeast A and B. Both of these breweries employ pure culture apparatus. The yeasts were typed as to growth requirements by the methods already described with results showing that:

Yeast A is similar to culture 7C43 (see Fig. 1) in that it requires biotin, pantothenate and inositol.

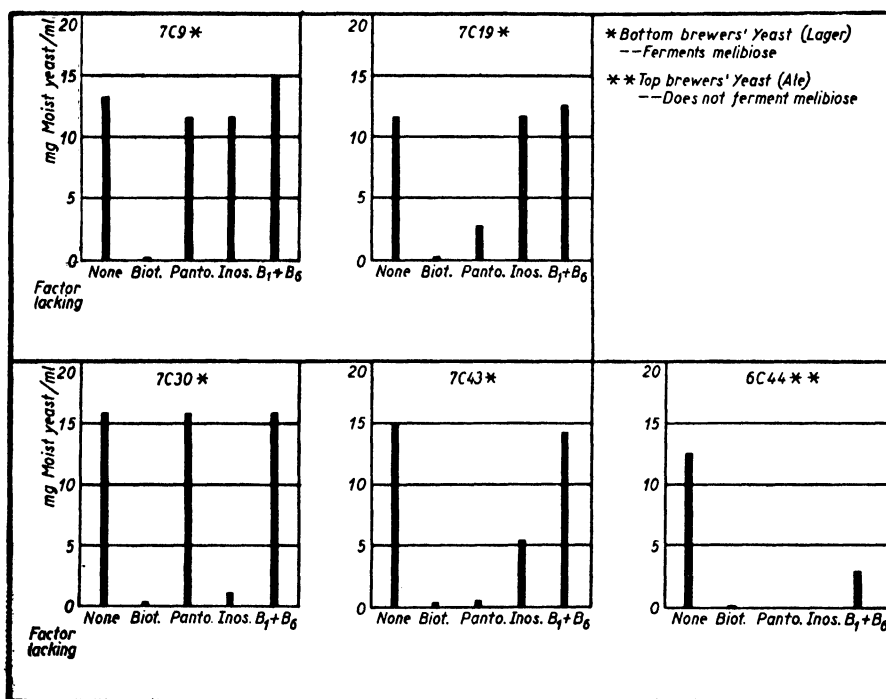


Fig. 1

Yeast B is similar to culture 7C9 (see Fig. 1) in that it requires only biotin.

*The Rate of Attenuation* was followed by daily measurements of the specific gravity. Fermentations were not agitated after the first 24 hours. One ml samples were removed each day and, after degassing, the specific gravity was determined in a specific gravity gradient tube<sup>10</sup>.

*The Reagents* were, in all cases, the best chemically pure grades available. The yeast extract was obtained from the DIFCO LABORATORIES INC.

## EXPERIMENTAL

For this phase of the work, some modifications of the medium of Table VI were

found desirable. The dextrose content was increased to 7%. Some modification was made in the buffer content. In Fig. 2 are shown the rates of attenuation of this medium (with and without bios factors) as compared with that of beer wort. Preliminary experiments had shown a faster rate of attenuation of wort; consequently this experiment included tests in which yeast extract or a mixture of bios factors was added to the basal synthetic medium.

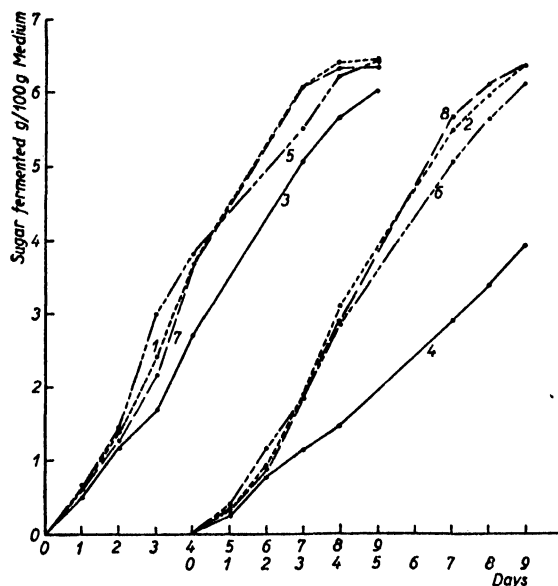


Fig. 2. Rate of Attenuation in Wort and Synthetic Medium Supplemented with Yeast Extract or Additional Bios Factors

Test	Medium (500 ml)	Pitching Rate Pressed Yeast mg/ml
1	Beer wort, 12° P	1.94
2	Beer wort, 12° P	0.97
3	* Synthetic medium (7 % dextrose)	1.94
4	* Synthetic medium (7 % dextrose)	0.97
5	* Synthetic medium (7 % dextrose) plus 1.00 g yeast extract	1.94
6	* Synthetic medium (7 % dextrose) plus 1.00 g yeast extract	0.97
7	** Synthetic medium (7 % dextrose) plus six bios factors	1.94
8	** Synthetic medium (7 % dextrose) plus six bios factors	0.97

\* No bios factors

\*\* Biotin, 0.25 mg; Calcium pantothenate, 5.00 mg; Inositol, 5.00 mg; Thiamin, 0.40 mg; Pyridoxine 0.40 mg; Nicotinic acid 4.00 mg.

From the course of the fermentations as shown in the graph, it can be seen that the addition of either yeast extract or the bios mixture, (biotin, pantothenate, thiamin, pyridoxine, nicotinic acid and inositol) causes an increase in attenuation rate so that the supplemented solutions are equal to wort. In Fig. 2, the extent of fermentation is indicated in terms of sugar fermented.

It is apparent that the necessity for supplementation of the synthetic medium is greater at the lower pitching rate. This, no doubt, is a reflection of the additional yeast growth required at lower pitching rates if a normal yeast crop and consequent normal rate of fermentation are to be developed. Since we were primarily interested in comparing beer wort and the synthetic medium, the lower pitching rate was employed to emphasize such differences in the experiments which follow.

#### *The Effect of Inositol*

Since it was apparent that the factor or factors responsible for the increased rate of attenuation of the supplemented medium could be found in the bios mixture, a series of tests were made in which the bios factors were singly omitted. As a matter of interest, the need for those bios factors originally present in the basal medium, *i.e.*, thiamin pyridoxine and nicotinic acid, was also studied in this series of tests. The results are

shown in Table VIII, from which it is apparent that inositol is the dominant factor for fermentation under these conditions. The omission of inositol alone caused a reduction in rate as marked as the omission of all of the bios factors.

TABLE VIII  
INFLUENCE ON ATTENUATION RATE OF OMISSION OF  
BIOS FACTORS FROM SYNTHETIC MEDIUM\*  
(PITCHING RATE = 0.97 MG PRESSED YEAST PER ML  
TEMP. = 12° C)

Bios Factor Omitted	Per Cent Sugar Fermented G sugar/100 g medium	
	3 days	6 days
None . . . . .	2.9	5.9
Inositol . . . . .	1.5	3.6
Pantothenate . . . . .	2.6	5.8
Biotin . . . . .	2.7	5.8
Thiamin . . . . .	2.8	5.5
Pyridoxine . . . . .	2.8	5.9
Nicotinic acid . . . . .	2.9	5.7
All bios factors . . . . .	1.8	3.8

\* Synthetic medium as shown in Table VI with the following changes: Dextrose, 70 g; Sodium succinate buffer, 100 ml; Biotin, 0.50 mg; Calcium pantothenate and Inositol, 10.0 mg per liter each.

### *Different Yeast Types*

It is apparent that the importance of inositol for normal attenuation in synthetic media might hinge on the specific bios type of the brewers' yeast culture employed, *i.e.*, an inositol effect might be expected by those yeasts which cannot synthesize this bios factor but might not be expected for others. Of the two pressed brewery yeasts available for this study, yeast A required biotin, inositol, and pantothenate, while

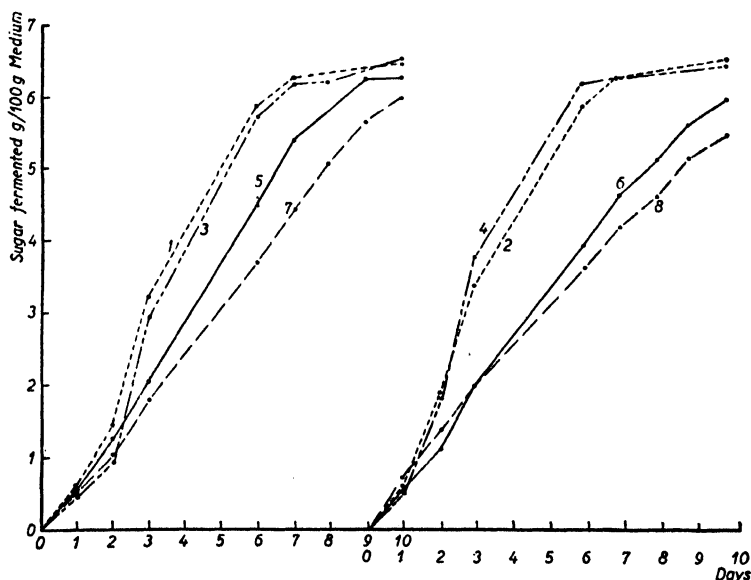


Fig. 3. The influence of inositol and pantothenate on the rate of attenuation by two different yeasts  
References p. 708.

yeast B required only biotin. In the series of experiments represented in Fig. 3, the two yeasts were compared. The rate of fermentation by each yeast in each of four solutions is here depicted. One solution contained all six bios factors (Test 1 and 2), one contained all but pantothenate (Test 3 and 4), one contained all but inositol (Test 5 and 6), and one contained no bios factors whatsoever (Test 7 and 8).

Test No.	Medium (500 ml)	Yeast (0.97 mg/ml)
1	*Synthetic medium with all bios factors	A
2	Synthetic medium with all bios factors	B
3	Synthetic medium with all factors except pantothenate	A
4	Synthetic medium with all factors except pantothenate	B
5	Synthetic medium with all factors except inositol	A
6	Synthetic medium with all factors except inositol	B
7	Synthetic medium without bios factors	A
8	Synthetic medium without bios factors	B

\* Synthetic medium with all bios factors as shown in Table VIII.

As the results show, inositol is necessary in the case of both yeasts and the lack of pantothenate is without significant effect. Thus the difference in bios type is not reflected under these experimental conditions. It should not be necessary to dwell at length on the wide difference between the conditions of this test and the conditions employed in the bios typing tests. It is probably sufficient to point out that, in the present tests, there is no more than an eight-fold multiplication of the yeast, whereas in the bios typing method, multiplication of the yeast inoculum may range from 500 to 1600 times. The difference in response to an inositol deficient medium is probably related to the inability of yeast to store inositol in excess of needs for multiplication and in the case of a yeast which can synthesize inositol, also inability to do so with sufficient rapidity under these experimental conditions.

*The Specific Gravity of the Synthetic Medium.* As previously noted, the gravity of the synthetic medium is considerably lower than that of wort both initially and during the course of fermentation. This is because the non-fermentable dextrins of beer wort have no counterpart in the synthetic medium. It was considered of interest experimentally to adjust the density of the synthetic medium to bring it in closer approximation to wort in this respect. Since well defined dextrins were not available, it was thought that lactose, a non-fermentable carbohydrate, might serve. Accordingly, 32.2 g per liter of lactose were incorporated in a synthetic medium. As shown in Fig. 4, the attenuation of this medium containing all of the bios factors was found to compare favorably with that observed in wort. Actually, the rate of attenuation of the synthetic medium is greater than that observed in wort. This is probably due to the fact that the synthetic medium contains dextrose as the only fermentable sugar.

*Influence of Other Bios Factors.* In order to determine whether inositol is the only bios factor required to reproduce the attenuation rate of wort, experiments shown in Fig. 4 are of interest. One is a fermentation of a synthetic medium to which none of the bios factors was added, and the other the fermentation of a medium to which only inositol was added. As may be seen, while inositol alone produces a pronounced stimulation, the other bios factors are also essential for development of a maximal rate.

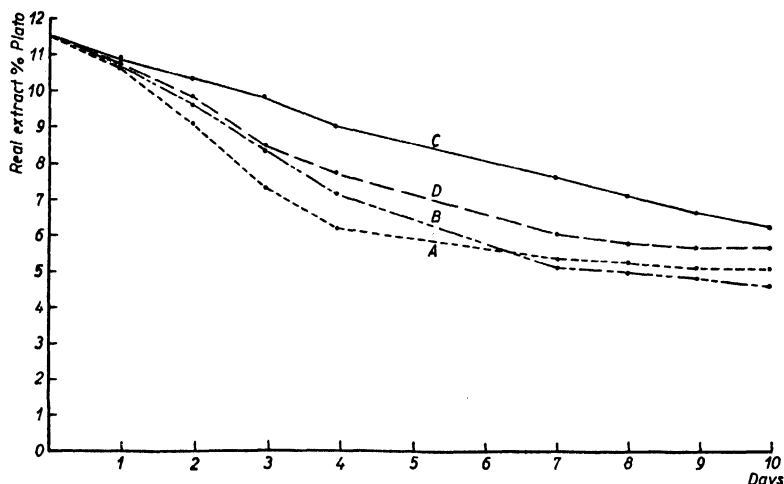


Fig. 4. Rate of attenuation of wort compared with synthetic media (with Plato adjusted by addition of lactose), yeast B.

Curve A. Synthetic medium with all bios factors as in Table VIII plus 16.1 g lactose per 500 ml.

Curve B. Brewery wort.

Curve C. Synthetic medium as in A but without bios factors.

Curve D. Synthetic medium as in A but with inositol as only bios factor.

**Effective Inositol Concentrations.** It is natural to inquire whether inositol might ever become a limiting factor in actual beer wort fermentation in the brewery. To answer this question, a series of tests were made with graded concentrations of inositol. It was found that a concentration of 1.5 mg of inositol per liter was adequate for the maximal effect. Several samples of brewery wort, separately analysed for available inositol by a yeast microbiological assay method<sup>10</sup>, gave values ranging from 35 to 50 mg of inositol per liter. Based on such values, it would appear that an inositol deficiency is not likely to occur in normal brewing practice. However, further investigation may well reveal the existence, at least under some circumstances (*e.g.*, due to varietal differences in barley or processing conditions) of antimetabolite substances acting as anti-vitamins. A close similarity between the structure of a recently introduced insecticide, hexachlorocyclohexane (gamma isomer) and inositol points to at least one possible relationship of this kind. Some tests already conducted have shown such an anti-inositol effect in the case of this gamma isomer.

#### DISCUSSION

Our observations in the third phase of the work conducted with conditions more closely approaching those of brewery fermentation represent only a beginning. The fact that inositol, in these experiments, appears to play a dominant role in attenuation, and the observation that this behaviour is not directly correlated with a growth requirement for inositol merit some consideration and explanation, if possible. One explanation that suggests itself is that inositol may be more than simply a growth factor. It may, in fact, play a specific role in fermentation under the experimental conditions employed. Such a role for inositol has not been previously reported, and there is a lack of any contributory evidence which would, at present, support such a concept.

References p. 708.



The explanation which seems more likely to be correct, in the present state of our knowledge, hinges on the relative rates of growth occurring in the three experimental yeast environments employed. One environment heavily emphasized growth, resulting in a multiplication of 500 to 1600 times, another minimized growth so that the yeast dry matter increased by only one-fifth of its initial weight during the tests. Furthermore, in the first case, only yeast growth was measured; and in the second case, only alcoholic fermentation. Finally, in the attenuation experiments, the number of multiplications of the yeast is intermediate, of the order of four to eight, and our measurements (of specific gravity) were essentially measurements of the rate of alcoholic fermentation. Under these conditions, the rate of attenuation should reflect both the increase in the number of yeast cells and the fermentative activity of the number of cells present at each interval. Furthermore, the resulting curves are very sensitive to relatively small deviations in either factor, *i.e.*, cell number or fermentation intensity, because the reading records the *cumulative* effect, over a period of time, of changes in the sugar substrate. It is apparent from the results that unless the yeast receives an exogenous source of inositol, it cannot, under these conditions, manufacture this compound rapidly enough to coordinate with its other metabolic activities even though it has the capacity to do so eventually.

It has been shown that yeasts are able to absorb or otherwise retain large quantities of thiamin, one of the other bios factors. There is evidence that the very high amounts of this bios factor accumulated by the yeast represent a substantial excess over its normal metabolic requirements. The biotin content of yeast also has been shown to vary rather widely, although not to the same extreme degree as thiamin. It is probable that inositol is in a different class in this respect. Yeast may not be able to store up inositol in quantities more than enough for its functional requirements. In general, inositol differs from the other bios factors in other ways. It forms a rather large proportion of yeast dry matter, 0.5%. It may also differ from the other bios factors insofar as it may be a structural element, *i.e.*, a compound of the phospholipid lipositol rather than a catalytic factor such as a coenzyme postulated for all of the other five bios factors.

Although the other bios factors do not seem to play a role in our test in which the attenuation of synthetic solutions was followed when the yeast was taken from the bios-rich beer wort and pitched in the synthetic solution, it is very likely that successive fermentation cycles of these yeasts in synthetic media would disclose a need for the other bios factors in accordance with the requirements found in this bios typing procedure.

Even though two different brewers' yeasts were employed in the present study, it does not seem justifiable to generalize regarding the influence of inositol until other yeasts have been examined; yeasts which have been produced under different conditions and which originate from other cultures. Another factor which might be mentioned in the above connection is that bios typing interpretations are based upon a growth over a prolonged period (41 hours). In a shorter period (24 hours), virtually all of the yeasts show a stimulation of growth due to each of the bios factors.

With respect to the fermentation studies at 30° C in the fermentometer, this has been found adaptable to practical uses. Measurements of fermentative activity of liquid brewery yeasts may be made by this technique. In this way, it is possible to assess the vitality and characteristics of pitching yeasts in a short time. This has, in fact, been done with considerable success.

Although these investigations admittedly were far from complete, there has already developed a most useful application of the bios typing technique. It is widely recognized that standard methods of yeast classification are not wholly satisfactory. Usual tests are not adequately sensitive to the differences known to exist between strains of yeast. All lager yeasts, regardless of suitability for brewing, would probably be classified as *S. carlsbergensis*, STELLING-DEKKER. By means of the bios typing technique, it is possible to separate strains of yeast otherwise considered to be equivalent by previous methods of classification. This faculty has, in fact, already been put to practical use in several instances.

We have, for example, been able to determine that a yeast culture, introduced into a particular brewery 10 years earlier, has remained constant and essentially pure. This was established by comparison of a stock culture which had been maintained in the laboratory with yeast from the brewery. In two other cases, it was established in this way that the original culture was no longer present, in one instance apparently due to adventitious contamination and in another, to a change of culture not at first reported to the investigator conducting the bios typing of the yeast.

The bios typing technique would be of even greater utility were it true that each particular bios type was uniquely associated with a yeast strain of singular characteristics. Unfortunately, this does not appear to be the case, no such invariable correlation having been observed. There are indications, however, that a practical correlation may be found; for example, one useful lager culture was encountered which required only biotin for growth; another culture, also useful, required biotin, pantothenate and inositol. It so happened that the first culture was characterized as being dustier than the second. When these two cultures were used in a brewery, one gradually replacing the other, it was possible to follow the purity of the incoming culture, thereby enabling correlation of practical observations with the specific yeast. It is expected that applications of this kind will be multiplied as further experience is obtained.

#### SUMMARY

In this investigation, an attempt has been made to study growth and fermentation of brewery yeasts, as far as possible, in solutions of known composition. Growth and fermentation were first studied separately at a temperature of 30° C and with conditions adjusted as to emphasize first growth under one set of conditions and then alcoholic fermentation under another. Finally, the combined process as it occurs in brewery fermentation was studied at lower temperatures, also using solutions of known composition. In the growth studies, it was found that lager yeasts of different strains showed marked differences in their growth requirements. These growth requirements were found to be relatively stable and could be used to identify certain specific strains of yeast. In the fermentation studies, yeast nutrients were combined to produce a medium in which the fermentation rate was equal to that obtainable with beer wort. In the third phase of the work, this medium was employed under conditions of brewery fermentation such that both growth and fermentation occurred and, in this instance, it was found that inositol played a very important role in determining the rate of attenuation. The significance of this finding was further investigated with different brewery yeasts.

#### RÉSUMÉ

Dans ces recherches nous nous sommes appliqués à étudier la végétation et la fermentation de levures de brasserie, autant que possible dans des solutions de composition connue. Au début la végétation et la fermentation furent étudiées séparément à une température de 30° C dans des conditions choisies de façon à accentuer d'abord la première végétation, puis la fermentation alcoolique. Finalement le procédé combiné se produisant au cours de la fermentation en brasserie fut étudié à une

température moins élevée, également dans des solutions de composition connue. Au cours des études de végétation on a trouvé que les diverses variétés de levure de fermentation basse sont nettement différentes en ce qui concerne leurs besoins végétatifs. Il a été établi que ces besoins sont relativement stables et qu'ils sont utilisables pour l'identification de quelques variétés de levure spécifiées. Au cours des études de fermentation, des substances nutritives ont été combinées afin de produire un milieu dans lequel la vitesse de fermentation était égale à celle constatée dans le moût de brasserie. Dans la troisième phase du travail ce milieu fut employé dans des conditions de fermentation en brasserie, telles que la végétation et la fermentation se produisaient; on a trouvé dans ce cas que l'inositol joue un rôle très important en déterminant la vitesse d'atténuation. L'importance de cette découverte a été examinée de plus près en utilisant différentes levures de brasserie.

### ZUSAMMENFASSUNG

Bei dieser Untersuchung wurde ein Versuch gemacht, das Wachstum und die Gärung von Brauereihefen soviel als möglich in Lösungen bekannter Zusammensetzung zu studieren. Wachstum und Gärung wurden zuerst einzeln bei einer Temperatur von 30° C studiert, einerseits unter Bedingungen, welche das erste Wachstum speziell förderten, andererseits unter solchen, bei denen der Nachdruck auf die Gärung gelegt wurde. Schliesslich wurde der kombinierte Prozess, so wie man diesem bei der Gärung in der Brauerei begegnet, bei niedrigeren Temperaturen studiert, wobei ebenfalls Lösungen bekannter Zusammensetzung gebraucht wurden. Bei den Wachstumsstudien wurde gefunden, dass untergärrige Hefen verschiedener Rassen deutliche Unterschiede ihrer Wachstumsbedürfnisse zeigten. Diese Wachstumsbedürfnisse zeigten sich relativ stabil und konnten zur Identifizierung gewisser bestimmter Heferassen herangezogen werden. Bei den Gärungsstudien wurden Hefenährstoffe zu einem Medium kombiniert, in welchem die Vergärungsgeschwindigkeit derjenigen der Bierwürze gleichkam. In der dritten Phase der Arbeit wurde dieses Medium unter den Umständen der Brauereigärung verwendet, in der Weise, dass sowohl Wachstum als Vergärung auftraten und in diesem Fall wurde gefunden, dass das Inositol eine sehr wichtige Rolle spielt, indem es die Vergärungsgeschwindigkeit entscheidend bestimmt. Die Bedeutung dieser Entdeckung wurde weiter an verschiedenen Brauereihefen untersucht.

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